Universidade de Aveiro Departamento de Química
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Produção de PHA por culturas mistas a partir de resíduos industriais

Industrial by-product valorization through mixed culture PHA production

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Produção de PHA por culturas mistas a partir de subprodutos industriais

Industrial by-product valorization through mixed culture PHA production

Dissertation submitted to the University of Aveiro to meet the requirements for the Degree of Master Biotechnology, performed under the scientific guidance of Prof. Luísa Serafim, Invited Assistant Professor at Department of Chemistry, University of Aveiro, and Dr. Paulo Lemos, Assistant Researcher at Department of Chemistry, Nova University of Lisbon

To my family and friends

jury

President

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acknowledgments To Prof. Luísa Serafim and Dr. Paulo Lemos

To staff at UA and FCT/NOVA laboratories.

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To my closest friends for being cheerful and also for being the most hard and helpful critics.

To my Parents … Thank you

palavras-chave Polihidroxialcanoatos, Culturas Microbianas Mistas, Alimentação Aeróbia Dinâmica, HSSL, Glicerol em bruto, Análise Comunidade Microbiana

resumo

Na perspetiva do desenvolvimento de processos industriais sustentáveis, é necessária a valorização e transformação dos subprodutos obtidos. A produção de polihidroxialcanoatos (PHA) com recurso a culturas microbianas mistas (MMC) é um dos processos possíveis para fazer essa valorização. Neste estudo, foi avaliada a possibilidade de usar dois subprodutos: licor de cozimento ao sulfito ácido (HSSL) e glicerol em bruto (CG), como matérias-primas para a produção de PHA para os quais foram selecionadas duas MMC em dois reatores descontínuos sequenciais (SBR1 e SBR2, respetivamente) através da imposição de condições de alimentação dinâmica aeróbia (ADF).

A MMC selecionada no SBR1, apesar de não ser capaz de utilizar a principal fonte de carbono presente no HSSL, os lenhosulfonatos, consumiu acetato e xilose acumulando dois biopolímeros: poli(3-hidroxibutirato-co-3-hidroxivalerato) (PHBHV) e biopolímeros de glucose (GB). Durante o período de seleção foi obtido um conteúdo máximo em PHA de 6,6% e um rendimento máximo de 0,49 Cmmol HA/Cmmol S.

A comunidade microbiana selecionada no SBR1 foi analisada com recurso a fluorescence in situ hybridization (FISH) e Denaturing Gradient Gel Electrophoresis (DGGE) demonstrando a predominância de *Alfaproteobacteria*, com os géneros *Paracoccus* e *Rhodobacter* a serem identificados, mas também a existência de *Betaproteobacteria*, *Gammaproteobacteria* e também bactérias pertencentes às classes *Flavobacteria, Bacteroides, Sphingobacteria*.

Quanto à MMC selecionada no SBR2, esta demonstrou ter capacidade de consumir tanto a fração de glicerol como a de metanol presente no CG, sendo que apenas o glicerol pareceu contribuiu para o armazenamento tanto de poli-3-hidroxibutirato (PHB) como de GB. Usando o CG, a MMC selecionada atingiu um conteúdo máximo de PHB de 37,4% e um rendimento máximo de 0,62 (Cmmol HA/Cmmol S) num ensaio de acumulação.

keywords

Polyhydroxyalkanoates, Mixed Microbial Culture, Aerobic Dynamic Feeding, HSSL, Crude Glycerol, Microbial Community Analysis.

abstract

In the perspective of the development of sustainable industrial processes, the recovery and transformation of its by-products are mandatory. The production of polyhydroxyalkanoates (PHA) by mixed microbial cultures (MMC) is one of the possible routes to obtain. This study evaluated the possibility of using two industrial byproducts: hardwood sulfite spent liquor (HSSL) and crude glycerol (GC), as feedstock for PHA production by a MMC selected in sequencing batch reactors (SBR1 and SBR2 respectively) using the aerobic dynamic feeding (ADF) strategy.

The MMC selected in SBR1, despite not being able to use the main carbon source of HSSL, lignosulphonates, revealed the capacity to consume acetate and xylose to accumulate poly (3-hydroxybutyrate-co-3-hydroxyvalerate) (PHBHV), and glucose biopolymer (GB). A maximum PHA content of 6.6% and a maximum production yield of 0,49 Cmmol HA/Cmmol S were obtained during the selection stage.

The analysis of selected microbial community of SBR1, using fluorescence in situ hybridization (FISH) and Denaturing Gradient Gel Electrophoresis (DGGE) techniques, revealed the dominance of *Alphaproteobacteria*, in which *Paracoccus* and *Rhodobacter* genus were identified, but also the existence of *Betaproteobacteria, Gammaproteobacteria* and bacteria belonging to the classes *Flavobacteria, Bacteroides Sphingobacteria*.

The culture selected by SBR2 despite showing the ability to consume both the glycerol and methanol fraction present in the CG, only glycerol appeared to contribute to both poly-3-hydroxybutyrate (PHB) and GB storage. Using CG, the selected MMC achieved a maximum PHB content of 37.4% and a maximum yield of 0.62 (Cmmol HA / Cmmol S) in an accumulation assay.

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ABBREVIATIONS

Acet	–	Acetate
AN/AE	–	Anaerobic/Aerobic
ATP	–	Adenosine triphosphate
CDW	–	Cell dry weight
CEN	–	European committee for standardization
CG	–	Crude glycerol
COD	–	Chemical oxygen demand
DGGE	–	Denaturing gradient gel electrophoresis
DO	–	Dissolved oxygen
Ɛ	–	Molar absorptivity
EU	–	European Union
6-FAM	–	6-carboxyfluorescein
FF/FAME	–	Free fatty acids and fatty acids methyl esters
FID	–	Flame Ionization detector
FISH	–	Fluorescence *in situ* hybridization
GAO	–	glycogen accumulating organisms
GB	–	Glucose biopolymer
GC	–	Gas chromatography
GHG	–	Greenhouse gas
Gluc	–	Glucose
Gly	–	Glycerol
HA	–	Hydroxyalkanoate
3-HAME	–	3-hydroxyalkanoate methyl esters
HB	–	3-hydroxybutyrate
3-HBME	–	3-hydroxybutyrate methyl ester
HDPE	–	High-density polyethylene
HHx	–	3-hydroxyhexanoate
3H2MB	–	3-hydroxy-2-methylbutyrate

3H2MV	–	3-hydroxy-2-methylvalerate
HO	–	3-hydroxyoctanoate
HPLC	–	High-performance liquid chromatography
HSSL	–	Hardwood Spent Sulfite Liquor
HV	–	3-hydroxyvalerate
IfBB	–	Institute for Bioplastics and Biocomposites
ISA	–	Ionic Strength Adjuster
LCA	–	Life cycle analysis
LCL	–	Long Chain-length
LS	–	Lignosulphonates
MCL	–	Medium Chain-length
Meth	–	Methanol
MMC	–	Mixed Microbial Culture
NADH	–	Reduced nicotinamide adenine dinucleotide
$NADP^+$	–	Nicotinamide adenine dinucleotide phosphate
NADPH	–	Reduced nicotinamide adenine dinucleotide phosphate
NREL	–	National Renewable Energy Laboratory
OLR	–	Organic Load Rate
OPEC	–	Organization of the Petroleum Exporting Countries
OUR	–	Oxygen uptake rate
PA	–	Polyamide
PAO	–	Polyphosphate-accumulating organisms
PBAT	–	Poly(butylene adipate-co-terephthalate)
PBS	–	Phosphate Buffered Saline
PC	–	Polycarbonate
PCL	–	Polycaprolactone
PE	–	Polyethylene
PET	–	Polyethylene terephthalate
PHA	–	Polyhydroxyalkanoate
PHB	–	Poly(3-hydroxybutyrate)
P(3HB-co-3HV)	–	Poly(3-hydroxybutyrate-co-3-hydroxyvalerate)

PLA – Polylactic acid

PP – Polypropylene

PUR – Polyurethane

q_sAcet – Acetate specific consumption rate

q_sGB – GB specific production rate

q_sGly – Glycerol specific consumption rate

q_sHA – HA specific production rate

q_sMeth – Methanol specific consumption rate

q_sXyl – Xylose specific consumption rate

rpm – Revolutions per minute

RT-PCR – Reverse Transcriptase–Polymerase Chain Reaction

S – Substrate

SBR – Sequencing Batch Reactor

SCL – Short Chain-length

SCOD – Substrate chemical oxygen demand

TCA – Tricarboxylic acid

T_g – Glass-transition temperature

T_m – Melting temperature

TOC – Total organic carbon

TPE – Thermoplastic Elastomer

TSS – Total Suspended Solids

UPGMA – Unweighted Pair Group Method with Arithmetic Mean

v – Volume

VFAs – Volatile Fatty Acids

VSS – Volatile Suspended Solids

w – Weight

WWTP – Wastewater Treatment Plant

$Y_{HB/S}$ – HB production yield

$Y_{GB/S}$ – GB production yield

$Y_{X/S}$ – Biomass production yield

1

INTRODUCTION

1.1 Background, identification of the problem and motivation

Human development has always been associated directly or indirectly with energy use. According to World Energy Resources 2013 survey, in 2011, fossil fuels amounted to an 82% share of world's primary energy supply [1]. From these, petroleum was the largest single source of energy consumed by world's population (accounted for 32% of energy consumption in 2010) exceeding coal, natural gas, nuclear sources and renewable resources [1, 2].

The oil crisis in the 1970s and 1980s, which resulted in long queues outside petrol stations and the drastic increase of oil price, acted as a warning for the necessity of reducing the dependence on such feedstock and progressively replacing them by renewable ones. In the following years, discussions about "peak oil" - the point at which the world's oil supplies go into irreversible decline – arose the need to seek for alternative sources. Nowadays, that oil reserves are almost 60% larger than 20 years ago and the production of oil has gone up by 25%, the "peak oil" issue is not making headlines any longer [1]. Despite oil depletion not being as eminent as expected a few years ago, oil is still a finite resource and this issue will inevitably return in the future. In addition, there are other reasons that substantiate this transition. As seen in the 1973 OPEC (Organization of the Petroleum Exporting Countries) oil embargo, the unequal distribution of reserves around the world (OPEC countries account for more than 70% of world's total reserves) can lead to political control over oil supply causing drastic increases in the oil prices and market instability [1]. Furthermore, the environmental concerns due to the high greenhouse gas emissions resulting from fossil fuels consumption are also important reasons that sustain the necessity of moving from the actual petroleum-based to a sustainable industry based on

renewable resources. In the United States for example, 79% of 2011 greenhouse gas (GHG) emissions comes from the combustion of fossil fuels [3].

In order to answer to such necessity, the "biorefinery" concept arose that is gaining increasing attention, As illustrated in **Figure 1**, the biorefinery of the future will be analogous to today's petrorefineries. According to NREL (National Renewable Energy Laboratory) it will "integrate biomass conversion processes and equipment to produce fuels, power, and chemicals from biomass" [4]. These will include low-value, high-volume products, such as transportation fuels (e.g. biodiesel, bioethanol); commodity chemicals; materials; high-value, low-volume products; and speciality chemicals, such as cosmetics or nutraceuticals [5]. By producing multiple products, a biorefinery can take advantage of the different biomass components and intermediates, maximizing the value created from the biomass feedstock. Industrial biorefineries have been identified as the most promising route to the creation of a new domestic biobased industry [4].

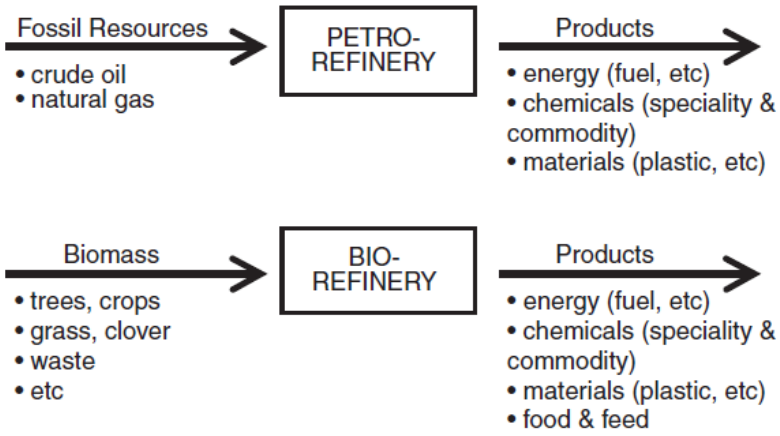


Figure 1 *- Comparison between petrorefinery and biorefinery [5].*

As one of the most relevant type of materials derived from petroleum, synthetic plastics, have a wide range of applications, from specialized products used in medicine to everyday products like packages. However, with a global production of 288 million tonnes in 2012, plastics accumulation has become a serious environmental problem [6].

Other than landfilling, there are two main methods to eliminate plastics from environment: recycling and incineration. Although a significant fraction of plastics produced worldwide is treated by these two methods, many others cannot, due to their

chemical characteristics or type of prior use [7]. In these cases, the utilization of polymers that degrade after their end-use is a worthy option. One class of such biodegradable polymers that possess similar physicochemical properties to some synthetic plastics (polypropylene, polyethylene) are polyhydroxyalkanoates (PHAs). Currently, the main obstacle to the replacement of common plastics by biodegradable polymers such as PHAs, is their high end price. In the current industrial production strategy, two of the most important factors that contribute to the high PHA production cost are: the maintenance of sterile conditions of the process and the pure substrates used. Thus, in order to overcome the contribution of these two factors to the production cost, new strategies using mixed microbial cultures (MMC) and renewable raw materials/waste residues as substrates have been subjects of several scientific studies [8-11]. Besides attempting to reduce PHAs production costs, another very significant advantage of this strategy comes up, that fits in the concept of biorefinery: the valorization of industrial residues with high organic load and low value that generally represent a disposal problem to the industry.

1.2 Objectives and strategy of research

The present work aims to contribute to tackle the concerns above stated by evaluating the possibility of valorize two different industrial by-products: hardwood spent sulfite liquor (HSSL) and crude glycerol (CG), by converting them into PHAs using MMC. More specifically, two sequencing batch reactors were fed with those residues and operated under aerobic dynamic feeding (ADF).

After selecting stable homogeneous high PHA storage capacity cultures in both SBRs, kinetic and stoichiometric parameters of the MMC were determined to evaluate the systems. Accumulation assays were performed to access the storage capacity of selected cultures. Finally, the systems were analyzed in order to identify and monitor the evolution of different groups of microorganisms present in the selected MMC and relate them with the storage capacity and kinetics.

2

STATE OF THE ART

2.1 Bioplastics

2.1.1 Definitions

Bioplastics comprise a whole family of materials that can vary considerably from one another and having different properties and varied applications. The term bioplastic itself can be vague and definitions do not always agree. IUPAC (International Union of Pure and Applied Chemistry) simply considers a bioplastic as a "Biobased polymer derived from the biomass or issued from monomers derived from the biomass" [12]. However, European Bioplastics, defines it as a "plastic that is either biobased, biodegradable, or features both properties" thus including the biodegradable plastics derived from petroleum, such as poly(butylene adipate-co-terephthalate) (PBAT) or polycaprolactone (PCL) [13]. However, in order to understand the full extent of the bioplastic definition is fundamental to bear in mind the meaning of the following terms: "biobased" and "biodegradable".

The term "biobased" means that the material or product is entirely or partially derived from biomass (e.g. corn, sugarcane, or cellulose) [12]. The major advantage of a biobased product is related with the reduction of the dependency on limited fossil resources by replacing them with a renewable feedstock. But also, the use of renewable resources, helps to reduce GHG emissions. Plants absorb atmospheric carbon dioxide and transform it into biomass. By using this biomass to produce biobased products, GHG are being temporarily removed from the atmosphere [14]. In general, one of the major benefits of biobased plastics is allowing closing the carbon cycle (**Figure 2**). This means that on a

biobased product, renewable energy can be recovered when recycling is no longer an option.

Companies can certify the "biobased carbon content" of their products as according to EU standard CEN/TS 16137:2011. The method of calculation is based on the ^{14}C content measurement, which in a product made from biomass is higher than in a fossil based material. The biobased plastics can also substantiate their claim with specific labels provided by organizations such as DIN CERTCO and VINÇOTTE [13].

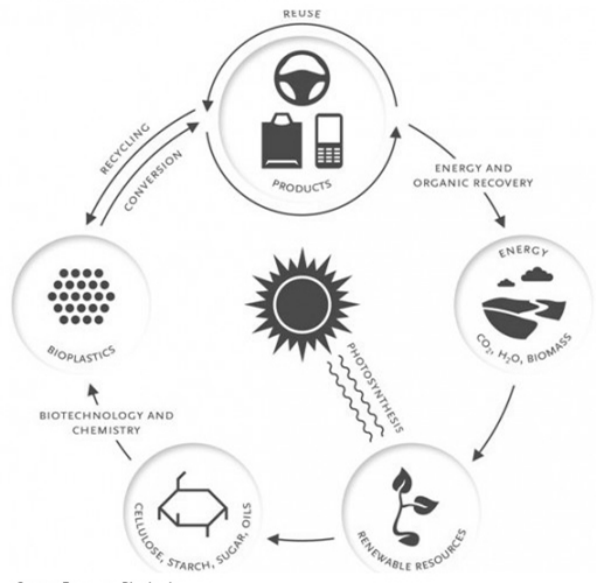


Figure 2 *- Idealized life cycle of biodegradable bioplastics produced from renewable resources [15].*

Regarding the term "biodegradable", its use is not always the most correct and sometimes can be misleading to the consumer. The European Committee for Standardization (CEN) defines a biodegradable plastic as "degradable material in which the degradation results from the action of microorganisms and ultimately the material is converted to water, carbon dioxide and/or methane and new cell biomass" [16]. Biodegradability does not depend on the origin of the plastic but is rather linked to its chemical structure. In other words, a biobased plastic may be non-biodegradable, while a fossil-based plastic can biodegrade. However, the use of this term is only informative if it is specified the environment and time by which biodegradation undergoes. In fact, all plastics (bio- and petroleum-based) are technically biodegradable, it is only the rate and the extent of degradation that varies. That is why European Bioplastics recommend the use of other term, "compostability" [17].

When a plastic is certified as compostable (EN 13432 or EN 14995), it means that the product can be diverted to the composting waste stream at the end of its life and treated in industrial composting plants under controlled conditions [18]. The European standard, EN13432, defines how quickly and to what extent a plastic must be degraded under industrial composting conditions to be called compostable (90% of the organic material must be converted into CO_2 within 6 months) [18].

2.1.2 Types of bioplastics

According to European Bioplastics definition it is possible to classify bioplastics in three groups (**Figure 3**).

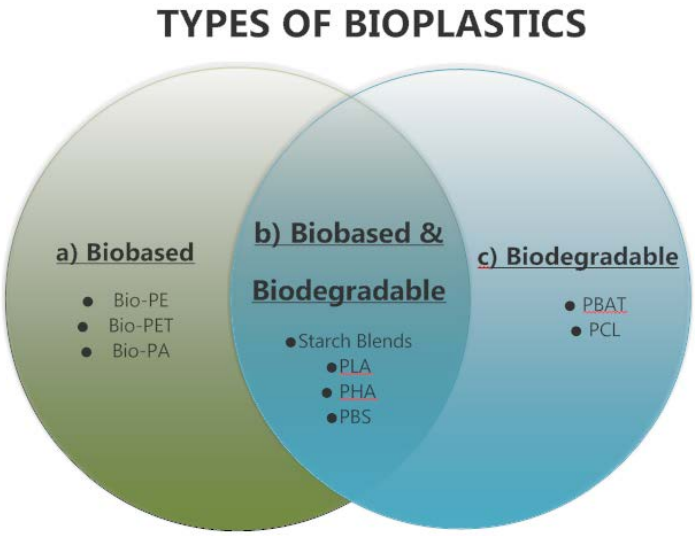


***Figure 3** - Bioplastics division in three main groups according to feedstock and biodegradability: a) Non-biodegradable biobased or partially biobased plastics; b) Biobased and biodegradable plastics; c) Biodegradable fossil-based plastics. Adapted from [13].*

a) Non-biodegradable biobased or partially bio-based plastics

This group of bioplastics is, by far, the one expected to account for the bulk of the bioplastics production capacity in the future. It includes commodity plastics like polyethylene (PE), polyethylene terephthalate (PET) or polypropylene (PP) that, aside fossil based production, can also be made from renewable resources like bioethanol [13]. As the value-added chain only requires small adaptation, and the

chemical and mechanical properties of the products are identical to their fossil counterparts, they are also referred to as 'drop-in' bioplastics. Braskem, Brazil, is one large-scale producer of Bio-PE (200.000 tons p.a.) while Toyota Tsusho, Japan, produces partially biobased PET (200.000 tons p.a.) [19, 20].

b) Biobased and biodegradable plastics

The production of biobased and biodegradable polymers can be classified into three subgroups based on the processes involved and also on the types of the polymers: (*i*) chemical polymerization of monomers derived from biological processes (e.g. polylactic acid (PLA)), (ii) the direct biosynthesis of polymers in microorganisms (e.g. PHA), and (iii) modification of natural polymers, (e. g., starch and cellulose) [21].

The technologies for the processing of natural polymers into a thermoplastic material by chemical, thermal and/or mechanical means are relatively well established and successfully commercialized. In contrast, PLA and PHA, which have been available on an industrial scale only for the past few years, are now emerging as the next generation of biobased and biodegradable plastics [21].

c) Biodegradable fossil-based plastics

This fossil-based bioplastics are a comparatively small group from which PBAT and PCL stand out. They are mainly used in combination with other bioplastics because of their capability of improving their performance in terms of biodegradability and mechanical properties [13]. However, partially biobased versions of these materials will surely be produced in the near future.

2.1.3 Market

Following the technical trial facilities at the beginning of the 1990s and the subsequent upscaling phase, industrial-scale capacity has now been achieved. The current market is characterized by strong diversification (there is a multitude of applications

ranging from packaging, catering products, consumer electronics, automotive, agriculture/horticulture and toys to textiles and a number of other segments) and high growth (more than 20 percent per year) [22].

A mix of internal and external market drivers explains the growth of the industry. The extensively publicized effects of climate change, the increasing dependence on fossil resources and current plastics disposal problem are the main external factors responsible by consumer's acceptance of bioplastics. From an internal perspective, bioplastics are efficient and technologically mature materials. For industry players, the advantages focus on advanced technical properties, which increase product attractiveness, potential cost reduction through economies of scale and the development of additional disposal options [23]. Significant financial investments have been made in production and marketing of bioplastics and the number of manufacturers, converters and end-users have been increasing steadily. Legal frameworks are also responsible for the stimulation of the market providing incentives for the use of bioplastics in several countries. Currently, there are about 115 manufacturers on the market, which, according to Institute for Bioplastics and Biocomposites (IfBB), accounted to a production capacity of approximately 1.3 million tonnes in 2012 (**Figure 4**). The same institution projects a bioplastics production capacity increase to over 5.7 million tonnes until 2016 [22].

The main responsible for this growth will be, by far, the biobased, non-biodegradable bioplastics group. As seen in **Figure 5**, leading this group is partially biobased PET, which was accounting for approximately 40 percent of the global bioplastics production capacity in 2012 and is expected to extend its leadership to an approximately 80% share in 2016.

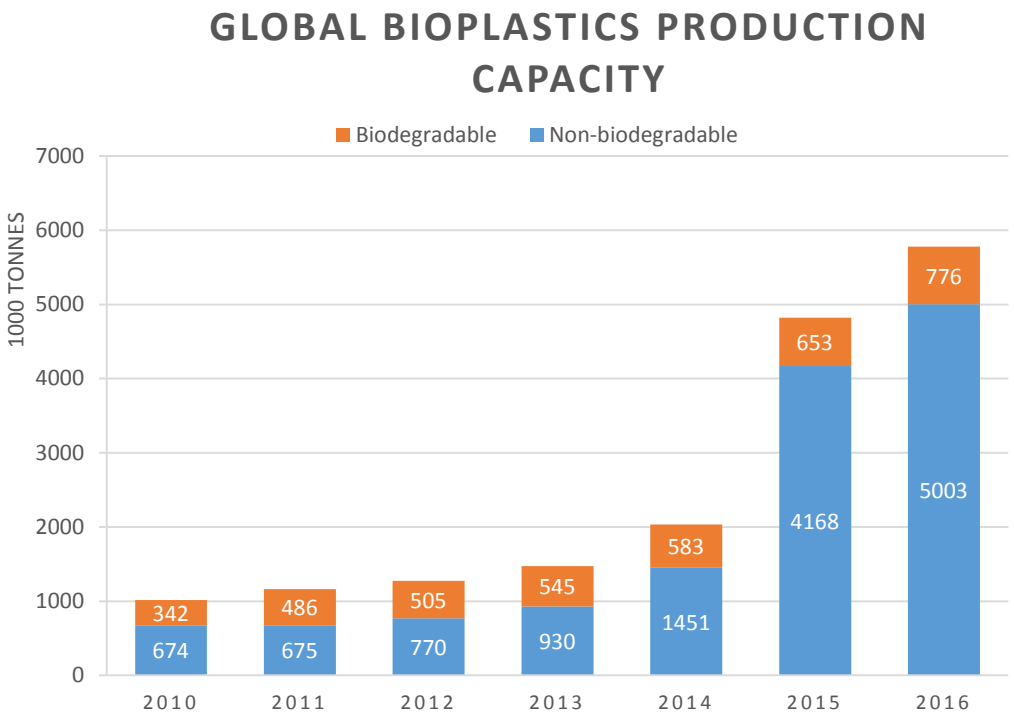


Figure 4 *- Global bioplastics production capacity. Adapted from [22]*

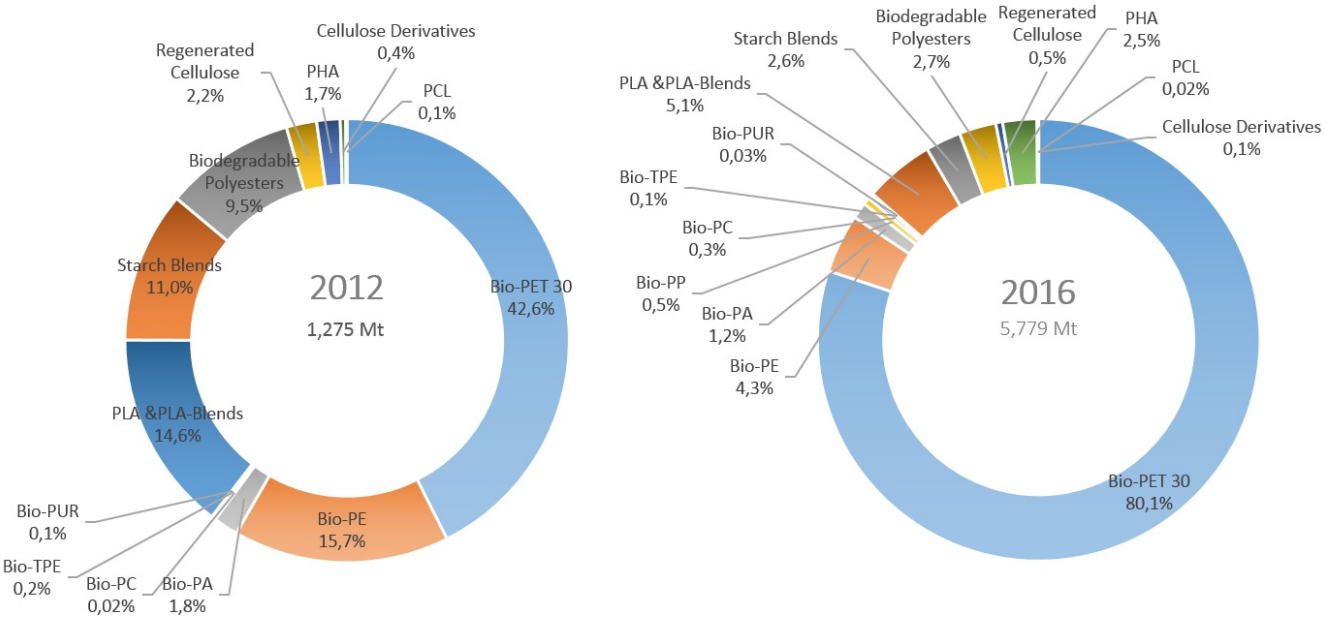


Figure 5 *- Material share of biopolymer production capacity in a) 2012 and b) 2016. Adapted from [22]*

But also biodegradable plastics are demonstrating considerable growth rates. Their production capacity is expected to increase by two-thirds by 2016. The leading contributors to this growth will be PLA and PHA, each of them accounting for 294.000 tonnes (+50 percent) and 144.000 tonnes (+550 percent) respectively [22].

2.2 Polyhydroxyalkanoates

2.2.1 Historical Outline

In **Figure 6** it is presented a historical timeline of the most important milestones regarding PHA discovery and research development.

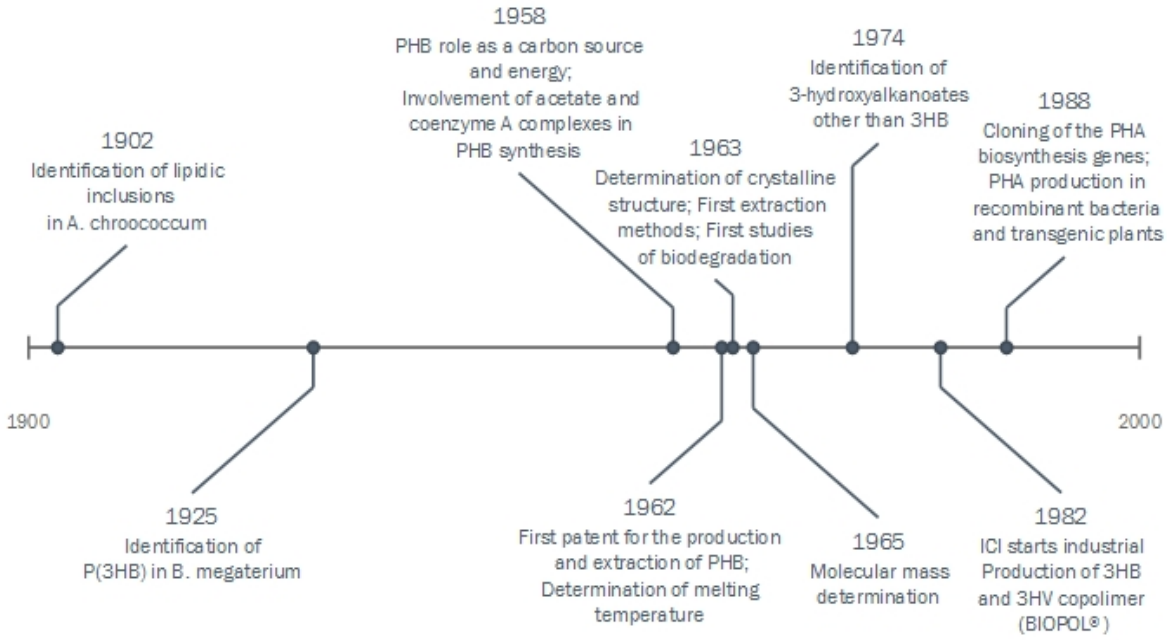


Figure 6 *- Historical timeline of PHA research milestones [7, 24]*

The discovery of PHA goes back to the early twentieth century, when in 1903, Meyer first observed in *Azotobacter chroococcum*, lipid soluble inclusions in chloroform [24]. Later in 1926, Lemoigne identified the chemical composition of similar inclusions in *Bacillus megaterium* as poly(3-hydroxybutyrate) (PHB) [25]. In 1958, Macrae and Wilkinson not only proposed that the existing *B. megaterium* PHB granules previously identified by Lemoigne, functioned as a source of carbon and energy for cell processes in order to avoid autolysis, but also correctly speculated on the involvement of acetate and coenzyme A complexes in the metabolic pathway leading to PHB formation [26].

By this time, 3-hydroxybutyrate (HB) was thought to be the only hydroxyalkanoate (HA) that formed this kind of microbial reserve polymer until Wallen and Davis reported in 1972, the isolation of a polyester with physical and chemical properties similar to those of

PHB from activated sludge [27]. Further analysis revealed the presence of 3-hydroxyvaleric acid (HV) and HB units as major components, and 3-hydroxyhexanoic acid (HHx), and possibly 3-hydroxyheptanoic acid units as minor components of the new compound [28]. This was the first report of a heteropolymeric PHA.

The commercialization of PHAs began in the 80s, through the company Imperial Chemical Industries (ICI), of England, who developed a process for the production and extraction of P(3HB-co-3HV) from substrates such as glucose and propionic acid [24].

With the onset of the molecular biology revolution during the late 1970s, new tools for biological research were provided that allowed the characterization of genes involved in PHAs biosynthesis. By the end of the 1980s, the genes that encoded enzymes involved in PHA biosynthesis were already cloned from *Cupriavidus necator* and shown to be functionally active in *Escherichia coli* and plants by recombination [24].

2.2.2 Physiology and biological function

It is believed that the PHAs can be synthesized by more than 30% of bacteria that inhabit the soil [29]. Besides that, many bacteria from activated sludge, marine environments and other extreme environments also have this capability [29]. The PHA content in most bacteria is low, ranging from 1 to 30 % cell dry weight (CDW). However, there are cases where they account up to 90% CDW [30].

When nutrient supplies are imbalanced, accumulation of PHAs is a natural way for bacteria to store carbon and energy for survival during prolonged starvation periods. These polyesters are accumulated when bacterial growth is limited by depletion of nitrogen, phosphorous or oxygen and an excess amount of a carbon source is still present [31]. The carbon sources are assimilated, converted into hydroxyalkanoate compounds and finally polymerized into high molecular weight PHAs and stored as water insoluble granules in the cell cytoplasm.

It is actually known that intracellular accumulation of PHAs enhances the survival of several bacteria under environmental stress conditions imposed in water and in soil [30]. In other words, PHAs confer an ecological advantage to bacteria that are able to synthesize

them. Bacterial cells with a high content of PHAs are more likely to survive longer than those that lack PHA or have a low PHA content because they can use their reserve materials when external carbon sources are scarce.

The number and size of granules of PHA per cell may differ depending on the PHA-producer microorganisms and their growth stage. In *C. necator* for example, 8-13 granules per cell with sizes ranging from 0.2-0.5 µm were detected [32]. These granules are refractive, allowing for its observation under phase contrast microscopy. The use of lipid dyes such as Sudan black (**Figure 7C**) and Nile blue (**Figure 7B**), allow for a differentiation of the remaining cellular material [33]. Also when thin sections of cells containing PHAs are viewed under transmission electron microscope (**Figure 7A**), the granules appear as electron transparent, discrete, spherical particles with clear boundaries.

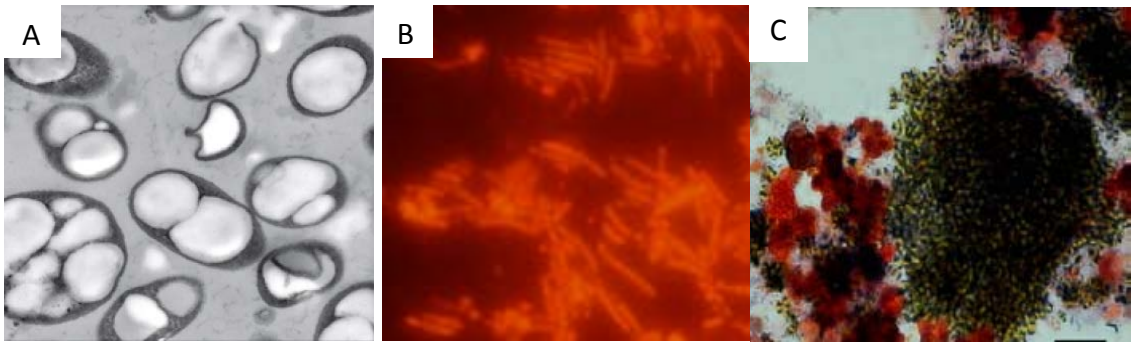
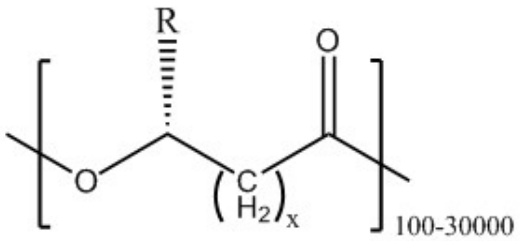


Figure 7 *- PHA granules observation under transmission electron microscope (A) and stained with nile blue (B) and sudan black (C).*

PHA granules are surrounded by a phospholipid monolayer membrane that contains associated proteins such as, PHA synthase, depolymerases, structural proteins and others. Unlike what was initially thought, PHA granules are maintained in an amorphous state *in vivo*. How microorganisms are able to prevent granule crystallization still remains unknown. The maintenance of PHA granules in the amorphous state *in vivo* is important because crystallized granules do not serve as a storage compound for the producing microorganism [24].

2.2.3 Structure and Properties

PHAs are a family of polyesters synthesized by several microorganisms and show rich structural variation. They can be composed by different monomer units known as hydroxyalkanoates (HAs) that can be aliphatic or aromatic [34]. Until today, there are more than 150 HAs described, although the 3-hydroxybutyrate (HB) is the most frequent one. In **Figure 8** is shown the general chemical structure of PHA.



x	*R*	*Monomer*
1	methyl	3-hydroxybutyrate (3HB)
1	ethyl	3-hydroxyvalerate (3HV)
1	propyl	3-hydroxyhexanoate (3HHx)
2	hydrogen	4-hydroxybutyrate (4HB)
3	hydrogen	5-hydroxyvalerate (5HV)

Figure 8 *- General structure of PHAs with some of the most common HAs. Adapted from [32].*

The *R* and *x* represented in **Figure 8** refer to the length of the side chain (may vary from a methyl to a tridecyl that can contain unsaturations, aromatic groups and halogenated elements) and the size of the alkyl group, respectively [35]. Both R and x determine the type of HA monomer unit.

When the total number of carbon atoms in the HA monomer unit is equal to or less than five, the HA is known as a short chain-length (SCL) monomer and the PHAs resulting from the polymerization of SCL monomers are known as scl-PHA. When the total number of carbon atoms in the HA monomer unit is at least six and up to 14, the HA is known as a medium chain-length (MCL) monomer and the PHAs resulting from the polymerization of MCL monomers are known as mcl-PHA. And finally, when the monomer has more than 14

carbons, the HA is known as long chain-length (LCL) monomer and the resulting PHA is known as lcl-PHA [36].

Their characteristics, variations in the length of the polymer and composition of available side chains lead to an innumerous variety of structures. In terms of monomer composition, PHAs can be produced as homopolymers, copolymers, and block copolymers. The structure and composition of the synthesized PHAs will depend on the type of microorganism, the raw material used as a carbon source, growth conditions as well as the metabolic pathways. This enables to obtain polymers that can widely vary in terms of physical properties, which allows a large set of potential applications. Whereas SCL monomers confer to the polymer a high crystallinity, tensile strength, and low elongation at break, MCL monomers, in other hand, confer elasticity, a reduction of crystallinity and melting temperature and improved elongation at break (**Table 1**).

Table 1 *- Physical and mechanical properties of PHAs with different monomer composition [32, 37, 38].*

	PP	*P(3HB)*	*P(3HB-co-10% 3HV)*	*P(3HB-co-16% 4HB)*	*P(3HB-co-10% 3HHx)*	*P(3HO-co-12% 3HHx)*
Melting temperature, T_m (°C)	168	177	140	150	127	61
Glass-transition temperature, T_g (°C)	-20	4	-1	-7	-1	-35
Tensile strength (MPa)	36	43	20	26	21	9
Elongation to break (%)	350	5	50	444	400	380
Crystallinity (%)	60	70	60	45	34	NA

NA - Not available

All these physical properties of PHAs closely resemble the properties of synthetic plastics currently dominating the market. Both PHAs and synthetic plastics are thermoplastics, moldable, and could be tailor-made for numerous applications ranging from stiff packaging goods to highly elastic materials for coatings [39]. In addition, PHAs have two qualities that sets them apart from conventional plastics:

- ***Biodegradability*****.** Microorganisms with the ability to degrade PHAs under aerobic or anaerobic conditions have been isolated from diverse ecosystems suggesting

that they can be assimilated and, thereby, preventing their environmental accumulation. Under aerobic conditions, PHAs can be readily degraded to CO_2, water and biomass, while under anaerobic conditions, PHAs are also degraded, although at a slower rate, to water, CO_2 and methane [40].

- ***Biocompatibility*.** This property indicates that PHAs do not cause a toxic effect when applied to a broad range of hosts. PHAs are immunologically inert and are only slowly degraded in human tissues [31]. Furthermore, the ideal biocompatibility of PHAs is underlined by the natural occurrence of 3HB and its low molecular weight oligomers and polymers in human blood and tissue [29]. This ability demonstrates the potential use of PHAs as biomaterials for biomedical devices.

2.2.4 Applications

Due to their diverse structures and properties, PHAs have penetrated different markets sectors. What started as an academic interest is now swiftly moving into the commercial field. Initially, PHAs were used to make everyday articles such as shampoo bottles and packaging materials. Currently, the most usual applications of bioplastics are as disposable items and biocompatible medical devices [41]. However, PHAs have a varied range of uses, covering virtually all applications of polypropylene. Next, are discussed the main sectors where PHAs are currently applied.

i) ***Industry***

Industry, in general, is one of the areas where the use of plastics assumes major proportions. The first consumer product made out of PHA was a shampoo bottle made of Biopol® launched in 1990 by Wella [42]. Since then, PHAs have been used mainly for the manufacture of short-lived products like packaging films, catering products, food packing, shopping bags and waste bags, and other disposable items such as razors, utensils, diapers, feminine hygiene products, [43, 44].

The gas barrier property of PHAs, specially P(HB-co-HV) make them also useful for applications in food packaging. The same property can be exploited to make coated paper and films, which can be used for coated paper milk cartons [41].

*ii) **Medicine***

Because of their properties, in particular, their biocompatibility, some bacterial PHAs (PHB, P(3HB-3HV), P(4HB), P(3HO) and P(3HB-3HHx)) are very attractive for several biomedical applications. As compiled by Philip et al. [41], they have been used for surgical sutures, in tissue engineering, in wound dressing, nerve conduits, carriers, scaffolds and drug delivery. However, the greatest contribution of PHAs to medicine has been in the cardiovascular area. Tepha Inc. specialized in manufacturing pericardial patches, artery augments, cardiological stents, vascular grafts, heart valves, implants and tablets, sutures, dressings, dusting powders, pro-drugs and microparticulate carriers using PHAs [44]. All these examples demonstrate how bacterial polymers are entering the biomedical field as promising and highly flexible materials.

It is important to note that the extraction procedures employed for PHAs to be used in medical applications need to ensure highly pure end-products, absence of halogenated solvents, non-degradation of the polymer and high efficacy [41].

*iii) **Others***

PHAs can also be used in other areas besides the above mentioned. For example, Zhang *et al.* [45] recently showed that 3-hydroxybutyrate methyl ester (3HBME) and mcl 3-hydroxyalkanoate methyl ester (3HAME) obtained from esterification of PHB and mcl-PHA could be used as biofuels. Brandl *et al.* [46] presented a bicycle helmet made of Biopol® and reinforced with cellulose fibers, which was shown to have a capacity of shock resistance comparable to commercial non-biodegradable plastic helmets. In agriculture, PHAs have been used in the controlled release of fertilizers, herbicides and insecticides [41].

2.3 Production of PHAs

PHAs are naturally synthesized by a large number of Gram-negative and Gram-positive bacteria belonging to at least 75 different genera [47]. These include bacteria such as *C. necator*, *Alcaligenes latus*, *Rhodococcus ruber*, *Syntrophomonas wolfei* and various species of *Pseudomonas* and *Aeromonas [48].* In some cases, these polymers can be accumulated intracellularly up to 90% CDW under conditions of nutrient limitation [49]. They can also synthesize a great variety of PHAs, depending on the carbon source and the culture conditions applied [50]. The *C. necator* and *P. putida* have been the most studied microorganisms for the production of PHAs [29] with *C. necator* (formerly known as *Alcaligenes eutrophus* and *Ralstonia eutropha*) being considered the model organism because of its great ability to accumulate PHB (up to 80-90% of its weight) from simple carbon sources [50]. In fact, this was the microorganism (named *Alcaligenes eutrophus* at that time) used in ICI industrial production process [51]. P(3HB) and P(3HB-co-3HV) were produced at a large scale from glucose and a mixture of glucose and propionic acid, respectively, in fed-batch bioprocesses [51]. The process of producing PHA involving this microorganism is divided into two phases: Initially *C. necator* was grown in a glucose-salts medium containing only the calculated amount of phosphate to support a desired amount of cell growth. When the phosphate became limiting, cells started to accumulate PHB from the supplied glucose [51].

There are other organisms such as *Alcaligenes latus* or mutant strain of *Azotobacter vinelandii* UWD, which grow rapidly and accumulate PHA simultaneously. *A. vinelandii* UWD can collect up to 70% of its dry weight [51] as PHA. Some microorganisms of the genus *Pseudomonas* can use n-alkanes, alcohols and alkanoates to grow and accumulate the PHA simultaneously, which also happens in the absence of nutrient limitation [51].

In addition to the natural PHA producers, it has been possible to obtain through genetic engineering techniques, genetically modified bacteria capable of producing PHAs. Genetic engineering has been applied either in naturally PHA producing bacteria, (as the case of *C. necator* in order to improve the productivity and the control over the monomer composition), but also in non-PHA producing bacteria with special emphasis on *E. coli* [29].

This last approach aims to overcome some of the main disadvantages of natural producers of PHAs such as: low growth rate, low optimal growth temperature and presence of PHA degradation pathways [50]. *E. coli* appears thus, as an organism of choice given that it grows quite fast and it's easy to lyse, allowing lower costs associated with the production process and purification step [35].

Although until now 300 bacterial species have been reported as having the ability to produce PHAs, only 75 were tested for production at industrial level. Until recently, industrial production of PHAs was exclusively based on the use of pure cultures of wild species or genetically engineered strains of *C. necator*, *A. latus*, *Aeromonas hydrophila*, *Bacillus spp., Pseudomonas putida, Pseudomonas oleovorans* and *E. coli* [44]. In **Table 2** are presented bacterial strains used for several industrial production processes.

Table 2 *- Bacterial strains used in the production of PHAs on a large scale and pilot scale [44].*

STRAIN	DNA MANIPULATION	PHA TYPE AND SCALE (T/A)	C-SOURCE	FINAL CDW ($g.L^{-1}$)	FINAL PHA (% CDW)	COMPANY
C. necator	No	PHB (10)	Glucose	>200	80%	Tianjin Northern Food, China
Alcaligenes latus	No	PHB(10-1000)	Glucose or Sucrose	>60	>75	Chemie Linz; btF, Austria; Biomers, Germany
E. coli	*ph*bCAB + *vgb*	PHB (10)	Glucose	>150	>80%	Jiang Su Nan Tian, China
Cupriavidus necator	No	PHBV (300-2000)	Glucose + Propionate	>160	>75%	ICI, UK; Zhejiang Tian An, China
Cupriavidus necator *Escherichia coli*	No *phbCAB*	P3HB4HB (>10 000)	Glucose + 1,4-BD	>100	>75%	Metabolix, USA; Tianjin Green Biosci. China
Cupriavidus necator	*phaC*$_{AC}$	PHBHHx (1)	Fatty acids	>100	>80%	P&G, Kaneka, Japan
Aeromonas hydrophila	No	PHBHHx (1)	Lauric acid	<50	<50%	P&G, Jiangmen Biotech Ctr,China
Aeromonas hydrophila	*phbAB* + *vgb*	PHBHHx (0.1)	Lauric acid	50	>50%	Shandong Lukang, China
Pseudomonas putida *P. oleovorans*	No	mcl PHA (0.1)	Fatty acids	45	>60%	ETH, Switzerland
Bacillus spp.	No	PHB (50)	Sucrose	>90	>50%	Biocycles, Brazil

CDW: cell dry weight; *vgb*: gene encoding *Vitreoscilla* hemoglobin; *phbCAB*: PHB synthesis genes encoding β-ketothiolase, acetoacetyl-CoA reductase and PHB synthase; 1,4-BD: 1,4-butanediol; *phaC*$_{Ac}$: PHA synthase gene *phaC* from *Aeromonas caviae*; *phbAB* encodes β-ketothiolase and acetoacetyl-CoA reductase.

Despite PHAs have already been targeted production at industrial level by several companies, its high price is the main reason why they are not yet widely used as substitutes for petroleum-based plastics. For this reason, ways of enabling a more competitive process are being sought. In this regard, the use of mixed microbial cultures (MMC) appears as a potential alternative as a means of producing PHAs at lower cost.

2.3.1 Mixed Microbial Cultures

Mixed microbial cultures are microbial populations with undefined composition, and whose structure results from the operating conditions imposed on the biological system. These conditions cause a natural selection of organisms with the ability to perform specific transformations of intracellular and extracellular components.

In PHA production, the selection of strains with high capacity to accumulate intracellular reserves results from the imposition of transient conditions. The production of PHAs by MMC was first verified in the biological phosphorus removal process, where cultures experience alternating conditions of anaerobiosis and aerobiosis (AN/AE) [52]. Other possible strategy for PHA production using MMC is the aerobic dynamic feeding (ADF). In this strategy, periods of excess of carbon alternate with substrate limitation, favoring the selection of the PHAs-accumulating communities under aerobic conditions.

An example of a MMC that experiences frequent changes in the availability of nutrients is activated sludge from wastewater treatment plants (WWTPs). Microorganisms developed in WWTPs are able to quickly store the available substrate and consume it during periods of lack of nutrients. This turns out to be a strong competitive advantage over the organisms without the same capacity [52, 53]

2.3.2 Pure Cultures vs Mixed Microbial Cultures

The current industrial production processes rely on the use of pure cultures that use expensive substrates such as glucose or propionic acid. This type of operation results in high investment needed (necessity for greater control of the operation and auxiliary

equipment for sterilization) and high production costs (in which substrates have an important role). Compared to pure cultures, MMC do not require sterile operation conditions and since the microbial population continuously adapts to the substrate, the selection of MMC with high PHA accumulation capacity under conditions of ADF has the advantage of allowing the use of a wide range of cheap complex substrates.

Taking this into account, it is estimated that the price of PHAs produced by mixed cultures can drop to about half the price of those produced by pure cultures [35]. In this context, PHAs production by MMC regarding the use of renewable raw materials/waste residues have been the subject of several scientific studies [8, 10, 53, 54].

When compared with pure cultures which maximum production amounts to more than 90 %CDW, in MMC has already been registered a PHA content of 89 %CDW with synthetic medium and 74.6 %CDW obtained from the real medium, such as fermented molasses [55, 56]. However, this approach must compete economically with other polymers production processes and with treatment processes for the same waste resource [57].

A Life Cycle Assessment (LCA) allows for the analysis of the process sustainability and determines its economic viability compared to processes already established. The number of published LCAs for PHAs is limited and only a few environmental analyses have been undertaken. It has recently been suggested, that PHA production using mixed cultures may be more favorable than using pure cultures in both economic and environmental terms [57]. Gurieff and Lant conducted a comparative LCA and a financial analysis of MMC PHA production against pure-culture PHA production and biogas production. The analysis was performed for industrial wastewater treatment technology, and the conditions used for mixed-culture PHA were compared with biogas production that uses the same waste resource. It was concluded that MMC PHA production was preferable to biogas production for treating the specified industrial effluent, and it was financially attractive in comparison to pure-culture PHA production. In addition, the mixed-culture and pure-culture PHA production processes had similar environmental impacts that were significantly lower than High-density polyethylene (HDPE) production [57].

2.3.3 PHA production mechanisms

As previously mentioned PHA accumulation by MMC can occur under anaerobic/aerobic (AN/AE) alternating conditions and under ADF. In the first case, two main types of microorganism are responsible for PHA accumulation: polyphosphate accumulating organisms (PAO) and glycogen accumulating organisms (GAO). PHAs synthesis plays an important role in their metabolism. In the absence of oxygen, carbon substrates are used for PHA synthesis and simultaneous consumption of glycogen occurs. Under aerobic conditions PAOs and GAOs consume the stored PHA for cell growth, maintenance and glycogen replenishment [52]. The amount of PHA accumulated by these groups of microorganisms is generally less than 20% [58].

In the ADF process, also known as “feast and famine”, microorganisms are subjected to short periods of excess substrate (feast) alternating with long periods of limitation of substrate (famine). After a long period of limitation of growth, an abrupt increase in substrate concentration in the presence of an electron acceptor and nutrients necessary for growth, microorganisms can have two different types of response depending on the nature of the substrate, biomass and operating conditions [59]. They either adapt to the new conditions by increasing the synthesis of active biomass, or they accumulate the substrate in the form of intracellular reserves.

The faster answer is the accumulation of intracellular reserves, because it requires less physiological adaptation of microorganisms. In this situation, the substrate is used almost entirely to the accumulation of intracellular reserves (about 70 % of the substrate). However, cell growth can become dominant if the exposure time to substrate is long enough to allow the physiological adaptation of microorganisms [60]. When the external substrate is depleted, the intracellular polymer acts as a source of carbon and energy. This process occurs when the final electron acceptor is oxygen or nitrate. The substrate and PHA variation that occur along the process of ADF are presented in **Figure 9**.

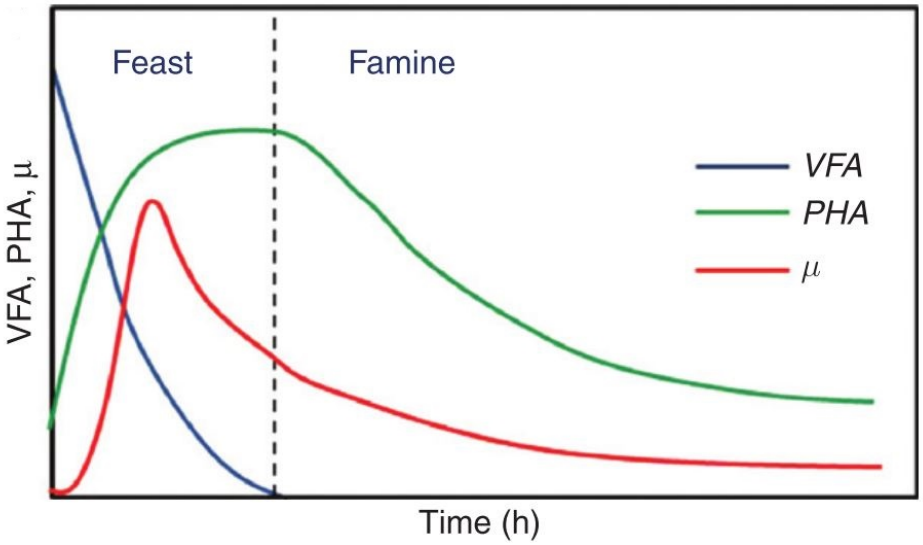


Figure 9 *- Mechanism for PHA storage by mixed microbial cultures under fully aerobic feast and famine [61]*

Since the concept of "feast and famine" process was first proposed by Majone *et al.* [62], many authors have been operating sequencing batch reactors (SBR) simulating these conditions [8, 63-66]. These studies led to a better understanding of the feast and famine process and subsequent optimization of reactor operating conditions. From that effort resulted MMC enrichment with a significant PHA storage capacity, being the selected culture able to reach a PHA cell content of up to 77% [67].

2.3.4 Metabolism

PHA biosynthesis has been well studied over the past years. However, the majority of the studies have focused on pure microbial cultures, while only few metabolic studies were reported for mixed cultures [68, 69]. It is assumed that PHA metabolism in mixed cultures is similar to that reported for pure cultures using the same carbon substrate [52]. These bacteria such as *Cupriavidus necator* and *Alcaligenes latus* use the Entner–Doudoroff pathway for carbohydrate catabolic degradation, resulting in the production of pyruvate, ATP, and reducing equivalents (NADH) [61]. When growth is not limited, pyruvate is converted to acetyl-CoA, which enters the tricarboxylic acid (TCA) cycle to be oxidized into CO_2 with simultaneous generation of anabolic precursors, energy, and reducing equivalents. With oxygen serving as final electron acceptor, these reducing equivalents are consumed to produce ATP through oxidative phosphorylation. However, when growth is

limited by, for example, a lack of an external nitrogen source, protein synthesis would slow down leading to accumulation of NADH and NADPH in the cell. This excess of reducing equivalents inhibit TCA cycle enzymes, thus directing acetyl-CoA to PHB synthesis (**Figure 10a**)[61]. Biosynthesis of PHB by this pathway starts with the condensations of two molecules of acetyl-CoA to acetoacetyl-CoA by β-ketothiolase (PhaA). This product is reduced by NADPH dependent acetoacetyl-CoA reductase (PhaB) to 3-hydroxybutyryl-CoA at the expense of the conversion of NADPH to $NADP^+$. This monomer is then incorporated into the polymer chain by PHA synthase (PhaC). An associated pathway involving PHA degradation helps regulate PHA synthesis and degradation [50].

Short-chain organic acids such as volatile fatty acids (VFAs) can also be percursors to the formation of different HA monomers by being activated to the corresponding acyl-CoA molecules (**Figure 10c**). When acetate is used as carbon source, two acetyl-CoA units give rise to PHB as described above. Propionate can be converted either to propionyl-Co-A or to acetyl-Co-A (by decarboxylation of propionyl-CoA) and, subsequently, two propionyl-CoA can form 3-hydroxy-2-methylvalerate (3H2MV), or one acetyl-CoA can be combined with one propionyl-CoA to form either 3HV or 3-hydroxy-2-methylbutyrate (3H2MB). Butyrate and valerate are converted directly to 3-hydroxybutyryl-CoA and 3-hydroxyvaleryl-CoA, which will, respectively, form 3HB and 3HV [61].

Three other metabolic pathways can originate precursors for PHA synthesis: β-oxidation of medium- and long-chain length fatty acids (**Figure 10d**); de novo fatty acid synthesis can produce intermediates for PHA production (**Figure 10b**); and alkanes can be oxidized to the corresponding alkanoate, which can be activated to the corresponding acyl-CoA molecule and, thus, can be used in PHA synthesis (**Figure 10e**).

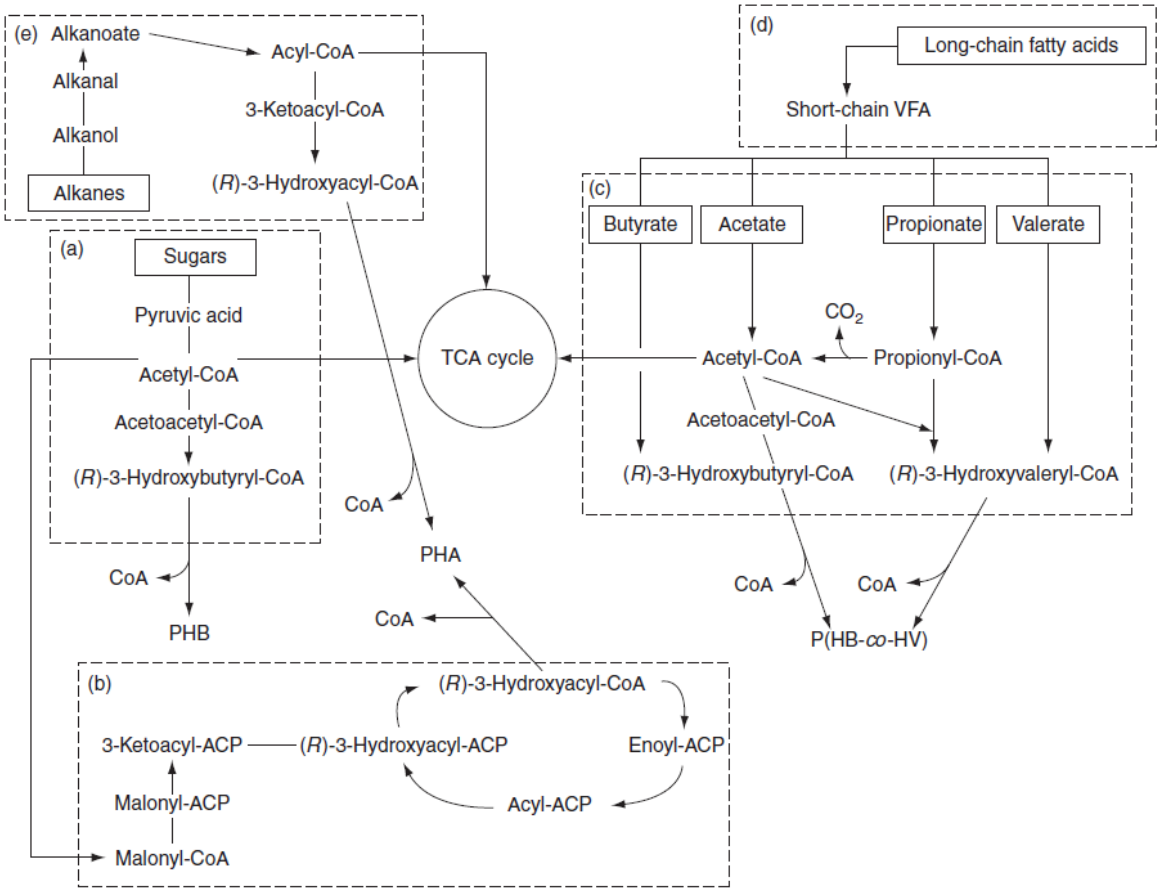


Figure 10 *- Five of the metabolic pathways involved in PHA synthesis: from sugars, through glycolysis or Entner–Doudoroff pathway (a) or de novo fatty acid biosynthesis (b); from fatty acids, directly (c) or through fatty acid β-oxidation (d); and from alkanes, through alkane oxidation (e) [61].*

2.3.5 Microbial Community Analysis

One critical factor on the development of a competitive process for PHA production with MMC is the selection of organisms with high storage capacity. During this step, it is important to monitor the population dynamics and identify the different groups of microorganisms involved in the feast and famine systems in order to relate them with the storage capacity and kinetics of the MMC. The characterization of the microbial populations selected under ADF conditions is still somewhat limited, with a high bacterial diversity being reported for most of these systems. In terms of general morphology, ADF conditions are usually known to select for floc-forming bacteria over filamentous organisms [61].

In the studies found in the literature, molecular approaches such as denaturing gradient gel electrophoresis (DGGE), fluorescence in situ hybridization (FISH) and reverse transcriptase–polymerase chain reaction (RT-PCR) were applied to identify the PHA accumulating organisms [8, 70-77].

Dionisi *et al*. [70, 71] identified PHA-accumulating organisms in an ADF reactor resorting DGGE and performing a 16S rRNA clone library. From the clones obtained, the most important organisms belonged to the *Betaproteobacteria* (*Thauera, Alcaligenes, Comamonas, Achromobacter*), although *Alphaproteobacteria* (*Xantobacter, Curtobacterium*) and *Gammaproteobacteria* (*Kluyvera, Pseudomonas, Acinetobacter*) were also present [70, 71].

FISH was also employed by Serafim *et al.* for the characterization of the microbial population in two SBRs under ADF conditions operated with acetate or propionate as sole carbon substrates [72]. Three main morphotypes were identified in both sludges: two belong to the *Alphaproteobacteria* class and the third to the *Betaproteobacteria* class. Bacilli affiliated to the genus *Azoarcus* and belonging to *Betaproteobacteria* were shown to be the main responsible for PHA storage [72].

Later in 2008, Lemos *et al*. [73] determined the identity of PHA storing bacteria selected under ADF conditions, using propionate and acetate as carbon source. This was done by applying RT-PCR on micromanipulated cells and confirming with FISH. Four genera, *Amaricoccus, Azoarcus, Thauera* and *Paraccoccus* were identified and their specific amount was determined by quantitative FISH. Variations in their relative abundance was shown in the different systems that could be explained due to the different VFAs used [73].

Microbial community analysis of SBRs operated under ADF conditions and fed with complex media was also performed. Moita and Lemos [8] used FISH analysis to monitor the composition of the microbial community fed with bio-oil resulting from the fast-pyrolysis of chicken beds. Dominance of *Betaproteobacteria* over the *Alpha* and *Gammaproteobacteria* was observed along the period of operation. *Thauera* genus was shown to be dominant although some genus composition variation was verified [8].

More recently, Albuquerque *et al.* [75] identified and quantified through a 16 S rRNA gene clone library and FISH analysis the microbial community of a fermented molasses-fed

SBR operated under ADF conditions and determined the carbon substrate-uptake preferences of different microbial populations. The microbial enrichment was found to be composed of the genera *Azoarcus, Thauera* and *Paracoccus* and the substrate preferences of each population were determined by microautoradiography-FISH [75]. Results showed that in the presence of multiple substrates, microbial populations specialized in different substrates were selected, thereby co-existing in the SBR by adapting to different niches. *Azoarcus* and *Thauera*, primarily consumed acetate and butyrate, respectively, while *Paracoccus* consumed a broader range of substrates and had a higher cell-specific substrate uptake [75].

2.3.6 Process layout

Depending on the type of substrate used as feedstock, PHA production by MMC can be operated in a two or three steps process (**Figure 11**).

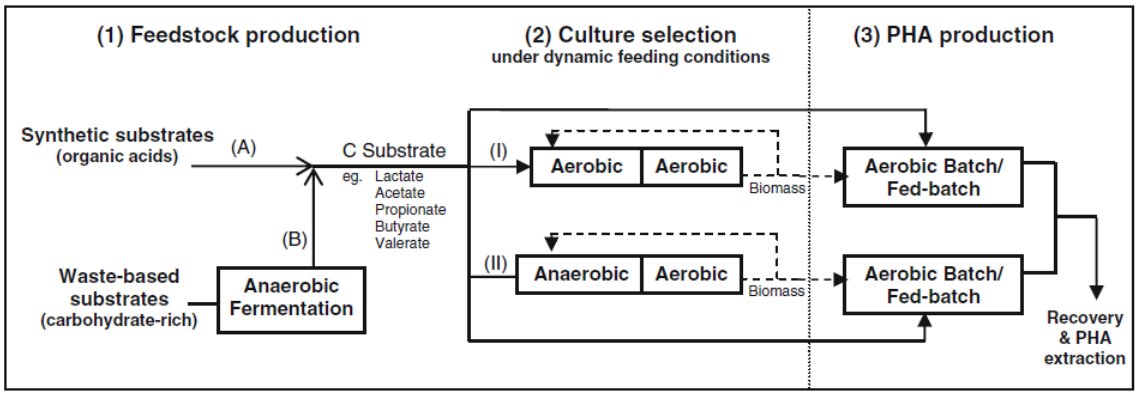


Figure 11 *- Three-step PHA production process by MMC using either synthetic (A) or waste-based substrates (B) and conducting culture selection using (I) Aerobic/Aerobic or (II) Anaerobic/Aerobic dynamic feeding strategies [52].*

When the feedstock used is mainly comprised by organic acids (e.g. acetate, propionate, butyrate, valerate or lactate), a two-step approach is usually applied [52]. This approach includes a step of selection of PHA-accumulating culture (step 2 in **Figure 11**) under aerobic or anaerobic/aerobic conditions, followed by a PHA accumulation (step 3 in **Figure 11**), in which the previously selected culture accumulates PHA in an aerobic batch or fed-batch regime [52]. Finally, the PHA produced in the accumulation step is then

extracted and purified. The physical separation of the culture selection stage from the PHA production phase allows for process optimization, since different optimal conditions were shown to be required in each step [78].

On the other side, when the feedstock is rich in carbohydrates, and since MMC submitted to feast and famine conditions are often referred as unable to store PHA from sugar-based compounds, a previous fermentation step (step 1 of **Figure 11**) is required [79]. In this extra stage, the objective is to transform carbohydrates into VFAs and other carboxylic acids that are more easily consumed by the selected culture. The overall setup results in a three-step process [71].

Most of the works that studied the production of PHAs by MMC submitted to ADF conditions were carried out in sequenced batch reactors (SBRs), operated with cycles of feeding, reaction, settling and drawn [80]. SBR are compact systems where the full feast and famine cycle may be performed in one single reactor, and the length of each phase may be varied, maintaining the same sludge retention time and the organic loading rate [52]. However Bengtsson *et al*. [11] tested, as an alternative to SBR, continuous reactors to produce PHA from fermented paper mill effluent. Also, as cited by Serafim *et al.*, the performance of two systems was compared by Albuquerque *et al.* for culture selection using fermented molasses as carbon source [52]. It was concluded that PHA content, polymer yield on substrate and specific productivity obtained were similar for both reactor configurations [52]. This result supports the possibility of using the facilities already existing in conventional wastewater treatment plants (except for the downstream) to produce PHA from industrial or municipal effluents [52].

2.3.7 Substrates

Most of the published work describes the production of PHA from simple substrates, such as sugars (glucose, fructose or sucrose) or carboxylic acids (acetic, propionic or butyric acid). However, *C. necator* may also use carbon dioxide to accumulate PHA and more complex compounds, such as 1,5-pentanediol, 1,7-heptanediol, 4-hydroxyvaleric acid or 4-valerolactone [7]. Some strains of *Pseudomonas* use alcohols such as methanol and

pentanol, as well as more complex molecules, such as n-alkanes, n-alkenes and n-alkanoates with long branched chains [7].

However, from a industrial point of view the use of these substrates mean a substancial share of the production cost accounting until 50% of total costs [81]. The price of PHA can be reduced substantially if cheaper, complex, substrates are used. Possible carbon sources are waste residues from several industries (food, agricultural, forest, paper). Several studies have been evaluating the use of such substrates as carbon sources for cultures selection under ADF conditions. Some examples include the use of: fermented sugar cane molasses [78], fermented palm oil mill effluents [82, 83], industrial wastewaters composed of formic acid, methanol and ethylene glycol [84], fermented paper mill wastewater [11], tomato cannery waste [54] and bio-oil resulting from the fast-pyrolysis of chicken beds [8].

Next, the two industrial by-products used in this work: HSSL and crude glycerol will be further detailed.

a) <u>HSSL</u>

The choice of the substrate should, whenever possible, meet the concept of the biorefinery. Among the different types of biorefineries, a lignocellulosic-based biorefinery could be the most successful one because of the abundance and affordability of raw materials, wide variety and good marketing of the bio-based products. Hardwood Sulfite Spent Liquor (HSSL) of *Eucalyptus globulus* is a by-product from acidic sulfite pulping process (**Figure 12**) in which a cooking liquor (composed by sulfur dioxide and bisulfite, as well as a counter ion) is used as a chemical treatment to obtain cellulose fibers (pulps) from wood [85].

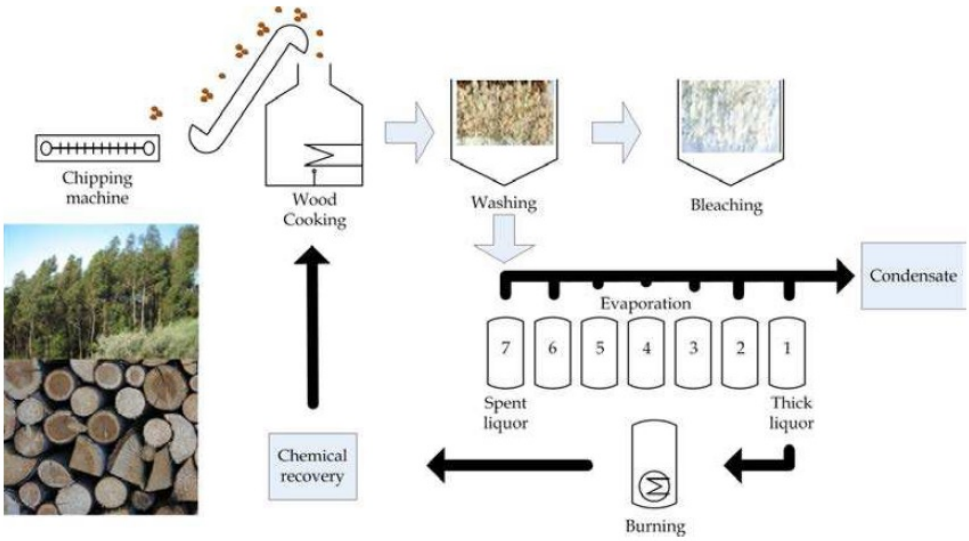


Figure 12 *- Scheme of acidic sulfite wood pulping process with Spent Sulfite Liquor release [85].*

This process is carried out in batch digesters under acidic conditions (pH 1–2) at 135–145 ºC for 6–12 h [85-87]. During pulping stage, the main reaction is sulfonation of lignin, which leads to its hydrolysis [87]. The composition of the resultant liquor, despite being highly dependent of the feedstock used, it is mainly composed by lignosulphonates (LS) and several sugars from degraded hemicelluloses. In addition, several potential fermentation inhibitor compounds can be present. These include sugar degradation products such as furfural and hidroximetilfurfural; lignin degradation products; compounds derived from extractives such as acetate and some phenolic compounds (e.g. gallic acid and pyrogallol); and heavy metal ions [86].

This by-product is produced in large amounts and is usually burned for energy and chemical recovery. However, due to its composition, it can be used to produce high value materials such as PHAs [88]. Using HSSL as feedstock to produce added value products fits well in biorefinery concept, once it decreases the dependence from fossil resources and improves the economic sustainability of pulping industries [89].

b) Crude Glycerol

Biodiesel is one of the promising alternative renewable fuels and has been viewed in the past few years with increasing interest. Consequently its production capacity was continuously growing over the last decade. Supported by governments to increase energy

independence and meet the rising energy demand, the biodiesel market is expected to reach 140 billion liters by 2016 [90]. However its relatively high production cost is pointed out as the main limiting factor for an even more significant production capacity growth. Utilization of the main biodiesel by-product, crude glycerol (CG), is one of the promising options for lowering the production cost. CG represents 10% (v/v) of the final ester, which according to the above-mentioned estimatives, will end up in a production of approximately 14 billion liters of CG in 2016 only [90].

There are several factors contributing to CG chemical composition: the type of catalyst used to produce biodiesel; the transesterification efficiency; recovery efficiency of the biodiesel; other impurities present in the feedstock; and whether the methanol and catalysts were recovered [90]. Purified glycerol is a high-value chemical with many applications, however CG requires an expensive refining process in order to achieve the necessary purity to be applied in food, pharmaceutical and cosmetics industries. Therefore, new applications for raw glycerol, have been sought in recent years, being his use as feedstock for industrial fermentations one of the most promising [66].

Production of PHAs from CG was already reported, although in most cases by applying pure cultures. Nevertheless, some studies have explored and proved the feasibility of the use of glycerol to produce PHA using MMC [66, 91, 92]. Moralejo-Gárate *et al.*, using synthetic glycerol as substrate, were able to enrich a PHA-producing microbial community under ADF conditions and subsequently achieve a PHA content up to 80% of CDW and a yield of 0.40 g PHB/g glycerol after a fed-batch PHA production step under growth-limiting conditions [91]. Dobroth et al. also enriched a stable mixed culture but used CG as carbon source instead of synthetic glycerol. However, although the authors reported a high PHB content, 62% CDW, the selected culture used exclusively the methanol fraction of the CG to produce PHB with a low polymer yield on substrate (0.10 g PHB/g methanol) [92]. More recently, Moita *et al*. were able to select a MMC able to consume both glycerol and methanol fraction present in the CG but with glycerol being be the only carbon source contributing to PHB production. A PHB content of 47% CDW and a productivity of 0.27 g X/L.d, was reached [66].

3

MATERIALS AND METHODS

3.1 PHA-accumulating culture enrichment

3.1.1 Feedstock

The first residue used was HSSL from magnesium based acidic sulfite pulping of *Eucalyptus globulus* supplied by Caima – Indústria de Celulose SA (Constância, Portugal). This liquor was supplied to a PHA culture enrichment system operated at University of Aveiro consisting in an open SBR reactor operated under ADF conditions called SBR1. HSSL composition is presented in **Table 3**.

Table 3 *- Composition of Eucalyptus globulus sulfite spent liquor [66, 89]*

		Concentration ($g.L^{-1}$)
Components	Lignosulphonates	78.2 ± 0.6
	Acetic Acid	8.2 ± 0.3
	Furfural	<0.1
	Ash	19.8 ± 0.2
Monosaccharides	D-Xylose	24.6 ± 0.5
	D-Mannose	8.5 ± 0.9
	L-Arabinose	7.8 ± 0.3
	D-Galactose	4.5 ± 0.1
	D-Glucose	2.3 ± 0.1
	L-Rhamnose	1.6 ± 0.3
	L-Fucose	0.4 ± 0.3

HSSL was collected from an inlet evaporator in a set of multiple-effect evaporators to avoid the presence of free SO_2 in liquor. A preliminary pretreatment described by Pereira *et al*. [93] was applied to HSSL to remove part of the most recalcitrant compounds. This pretreatment consisted in pH adjustment to 7.0 with 6M KOH followed by aeration with compressed air for 2 h per liter of HSSL processed. Then, the liquor was centrifuged for 1 hour at 5000 revolutions per minute (rpm) and the precipitated colloids were filtered off using a 1 µm glass microfiber filter. Finally, the total chemical oxygen demand (COD) of pretreated HSSL was determined (192.5 gCOD L^{-1}).

The other residue used in this research was CG which was used as feedstock to a similar PHA culture enrichment system operated FCT/UNL. This system was called SBR2. The CG used was obtained from an industrial biodiesel manufacturing plant Sovena (Almada, Portugal). Multiple vegetable oil sources are used by this industry to produce biodiesel and the CG formed was collected right after biodiesel production and before any purification step. This residue was then fed to the culture selection system directly and without any pretreatment. The CG was mainly composed by glycerol (71.66% g C/ g TOC) and methanol (25.69% g C/g TOC), but also contained a small fraction (2.58% w/w) of free fatty acids and fatty acids methyl esters (FFA/FAME) [66].

3.1.2 Inocula

The inoculum of SBR1 was obtained from a previous SBR fed with HSSL that has been continuously operating since January 2013 under aerobic dynamic feeding conditions. The inoculum was biomass purged from this reactor kept at 4°C. In order to eliminate potential anaerobic microorganisms and ensure an initial long famine phase, a previous aeration period of 24h was applied before inoculation.

SBR2 was inoculated with a previously acclimatized PHA-accumulating mixed culture, in this case, to CG [66] that was also subjected to a 24 h aeration period.

3.2.2 Reactor operation

In this study, sequencing batch reactors (SBR1 and SBR2) were operated, in order to select stable PHA-storing populations using the mentioned feedstocks. The reactors working volume was 1.5 L in both cases and were operated under aerobic dynamic substrate feeding, during which alternating feast and famine phases were imposed in each cycle.

The SBR1 reactor (**Figure 13**) operated on the following 8h cycle scheme: 15 min of feeding, in which 500 mL of fresh medium was pumped to the reactor (Watson – Marlow SCI 400 pump); 420 min of reaction, where the medium was kept aerated and stirred; 30 min of settling after agitation and aeration switched off; and 15 min of medium withdrawing (reactor volume was removed until a final volume of 1000 mL by a Watson – Marlow Pump 101 F/R). The biomass concentration in the bioreactor was maintained by purging 300 mL/d of reactor medium during the reaction phase. SBR1 worked with a hydraulic retention time (HRT) of 1 day and a sludge retention time (SRT) of 5 days. Reactor stirring, aeration and feeding and withdrawing pumps were controlled with timers. Dissolved oxygen concentration and temperature were measured with Oxygen meter Transmitter M300 (Mettler-Toledo Thornton, Inc). The system worked without pH and temperature control, although their values were monitored (Crison-PH 28 P). To prevent foam formation, diluted silicone anti-foam (1:20) was manually added when excessive foam was observed. The reactor was cleaned on a daily basis in order to prevent excessive biofilm formation on the reactor walls and electrodes.

SBR1 cycles were periodically monitored by taking samples across the entire reaction period and the evolution of biomass, acetic acid, xylose, LS, ammonium, COD and PHA content was analyzed. Biomass of the analyzed cycles was also collected at the end of feast phase for microbial characterization.

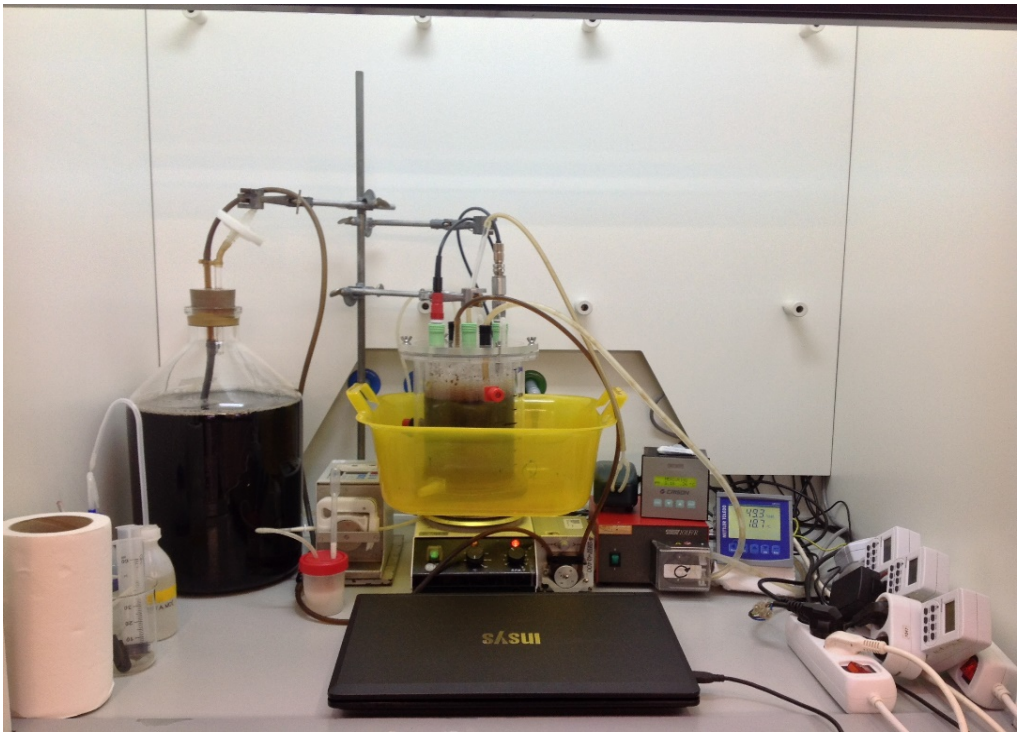


Figure 13 *- SBR1 bioreactor along with feeding vessel; aeration, feeding and withdrawing pumps; oxygen/temperature and pH meters placed in a laboratory fume hood.*

In SBR2 (**Figure 14**), each 24h cycle consisted of four periods: 8 min of fresh medium fill (750 mL); 1421.5 min of reaction under aerobic conditions; 10 min of settling and 0.5 min of reactor medium withdraw. HRT was kept at 2 days and a peristaltic pump (Watson Marlow SciQ 323) was calibrated to purge 300 ml of the reactor medium at the end of the aeration period in order to keep a SRT of 5 days. Air was sparged (1 L/min) through a ceramic diffuser and the pH was kept between 8.0 and 8.4 with 1M NaOH and 0.5M HCl and the reactor stood in a temperature-controlled room (20-23 °C). Reactor stirring (400 rpm), aeration, feeding, withdrawing and pH pumps were controlled by BioCTR software that also allowed the on-line measurement of pH and dissolved oxygen values.

As in SBR1, samples of several SBR cycles were taken and analyzed for the evolution of biomass, methanol, glycerol, ammonium, glucose biopolymer (GB) and PHA content.

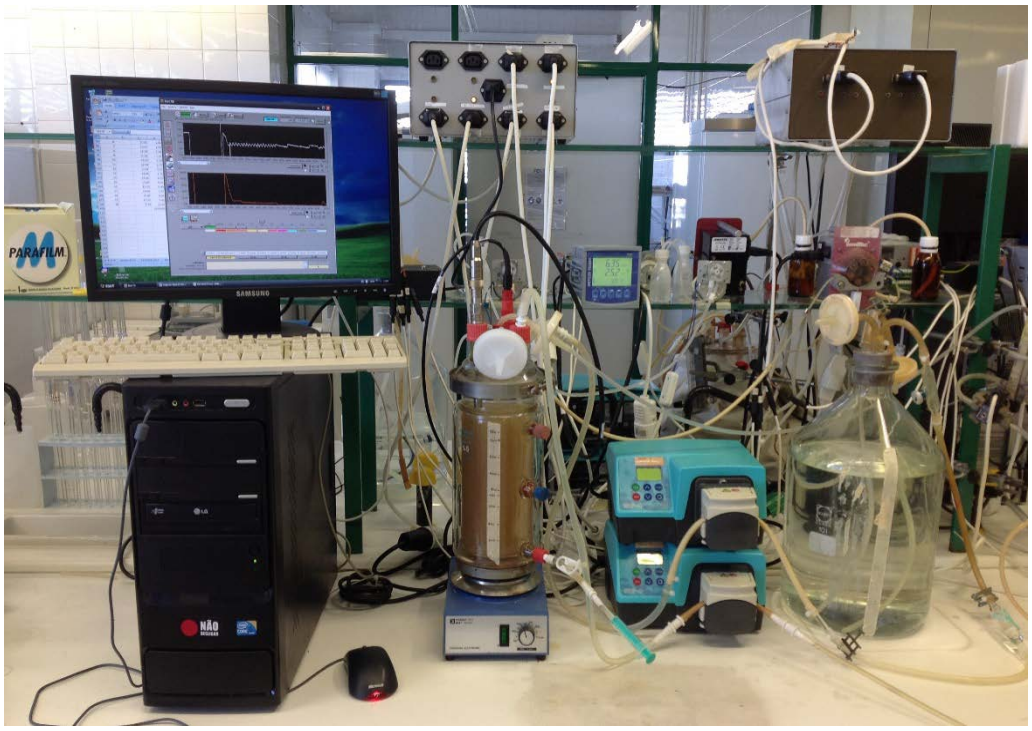


***Figure 14** - SBR2 bioreactor along with mineral solution and CG vessels; oxygen/temperature and pH meters; feeding, withdrawing and acid and base pumps controlled by software.*

3.2.3 Culture Medium

The HSSL used as substrate in SBR1, was diluted with a mineral solution prepared with distilled water (1:17), in order to achieve an organic loading rate (OLR) of 17 $gCOD.L^{-1}.d^{-1}$ in the SBR and supplemented with salts needed for microbial metabolism. The final medium composition is listed in **Table 5**. To inhibit nitrification, thiourea (400 $mg.L^{-1}$) was added. The pH of the medium was adjusted to 7.0 and the medium was autoclaved for 20 min at 121 °C. Phosphate salts were prepared separately, in order to avoid an irreversible precipitation with magnesium salts during sterilization and were added to the feeding flask under sterile conditions after sterilization.

In SBR2, CG and mineral nutrients solution were not sterilized and were fed separately to the reactor. At the beginning of each cycle the reactor was fed with 3.1 mL of CG (30 CmM) and 750 mL of mineral solution. The mineral nutrients solution was prepared in tap water and its composition is shown in **Table 5** which included nitrogen and phosphorus source to keep a C/N/P ratio at 100:8:1 (on a molar basis). Thiourea (10 $mg.L^{-1}$) was added to inhibit nitrification.

Table 5 - *Composition of mineral solutions of SBR1 and SBR2 [66, 88].*

Compound	Concentration ($mg.L^{-1}$)	
	SBR1	**SBR2**
KH_2PO_4	16	33
K_2HPO_4	64	63
$MgSO_4 \cdot 7H_2O$	160	-
$CaSO_4 \cdot 2H_2O$	80	-
$FeCl_3$	20	-
$Na_2MoO_4 \cdot 2H_2O$	8	-
NH_4Cl	160	260
CH_4N_2S	400	10

3.2.4 Sampling

In both reactors, samples for analysis started to be collected immediately after feeding (t = 0 min), and then, in SBR1 at intervals of 1 hour during the entire cycle, and in SBR2 at increasingly spaced time during the first 8 hours of the cycle. Samples were further centrifuged at 14000 rpm for 5 minutes in order to separate the pellet from the supernatant and both were stored at -20 °C. For SBR1, the pH of supernatant was measured (Hanna Instruments, HI 9321 Microprocessor, pH meter). Dissolved oxygen (DO) and pH in both reactors were recorded at the time of the sample collection.

The supernatant was used to measure substrates, ammonium, lignosulphonates concentration (only for SBR1) and COD (only for SBR1). The pellet was used to quantify glucose (stored as biopolymer) and PHA.

3.2 Accumulation Assays

In order to access maximum PHA accumulation capacity, storage yield on substrate and production rate of the cultures enriched in the SBR accumulation assays were performed for both systems.

In the case of SBR1, a single accumulation assay was performed at the 58th day of operation. In this case, the assay was performed in the SBR reactor itself by adding feed to the system in a pulse-wise manner to avoid potential substrate inhibition. The decision of adding a new pulse was based on the DO profile. Once the carbon was depleted and the DO increased abruptly, aeration and stirring were turned off to allow biomass to settle, and medium replacement was preceded. The feeding had the same composition and it was in the same proportion as the main reactor. Samples were collected every 15 min along with the pH and DO values.

For SBR2, the accumulation experiments were carried out in a separate 900 ml working volume reactor. 450 mL of sludge was collected from SBR2 at the end of the famine phase and washed twice with mineral solution (without any carbon and nitrogen source) before the beginning of the accumulation assays. In order to maximize storage, the accumulation assays were carried out under ammonia limitation. The C/P ratio however, was maintained the same as in the SBR2. Mineral medium was supplied once at the beginning of the test while CG was added to the system in successive pulses of 30 CmM to avoid substrate inhibition. The decision of adding a new carbon pulse was based on DO in the reactor and confirmed by the oxygen uptake rate (OUR) of the culture. When the DO value increased significantly and the OUR decreased, a new pulse of carbon was immediately added. A ceramic diffuser supplied air and magnetic stirring provided mixing. The pH and OUR were monitored over time. The determination of OUR was achieved by recirculation of the reactor medium through a respirometer (using a peristaltic pump), where mixing was provided by magnetic stirrer and an oxygen probe was inserted. Recirculation was stopped at given intervals and the decrease in dissolved oxygen concentration in the respirometer was registered and used to determine the OUR. The accumulation assays were conducted in a temperature controlled room (20–23°C).

3.3 Analytical Methods

3.3.1 Carbon Sources Analysis

Acetic acid and xylose of SBR1 samples were measured by HPLC in order to determine the evolution of concentration of these substrates along the experiments. 800 µL of the samples were filtered at 10000 rpm for 20 minutes, and injected (auto sampler – HITACHI L2200) in an ion exchange column Knauer Eurokat® of 10 mm, connected to a pump (HITACHI L2130) and refractive index detector (HITACHI L2490). The column temperature was 40 °C (Oven Gecko 2000) and the eluent was H_2SO_4 0.01 N at a flow rate of 0.4 mL/min at room temperature. A calibration using standard curves for acetic acid (0–1.5 g L^{-1}) and xylose (0–3 g L^{-1}) was applied [89].

The determination of LS content was performed according to Restolho *et al*. [94]. The measurement was performed using a UV Spectrophotometer (Shimadzu UVmini-1240) at 273 nm, after a dilution of 1:200. The lignosulphonates concentration was calculated resorting to the Beer-Lambert law, using a $\varepsilon = 7.41\ g^{-1}cm^{-1}$ [89].

Carbon sources present in SBR2 feeding (glycerol and methanol) were determined by GC. 1.3mL of samples were firstly filtered using a 0.2 µm (Whatman) membrane at 13000 rpm for 10 min. To each sample was added 100 µL of 1,4-Butanediol to act as internal standard with a final concentration of 0.2 mg/mL. 1 µL samples were injected into a gas chromatograph carrying a Bruker BR-SWAX column (30 m × 0.53 mm × 1 µm) and using H_2 (3 mL/min) as carrier gas. The injection was splitless and injector temperature was set to 290°C. The oven temperature program started with 80°C as initial temperature; then 10°C/min until 200°C; and kept at that temperature for 10 min. Coupled to the GC was a Flame Ionization Detector (GC-FID, Bruker 400-GC) at 280°C. Methanol and glycerol concentrations were obtained resorting to a calibration curve obtained through methanol and glycerol successively diluted standards.

3.3.2 Biomass Concentration

Biomass concentration for both systems was determined using total suspended solids (TSS) and volatile suspended solid (VSS) procedure described in Standard Methods [95]. 5 mL of sample were filtrated using previously dried and weighted filters (Cellulose Acetate Filter, 0.2 µm pore size, Sartorius) with vacuum filtration. The membranes were placed in evaporating dishes previously cleaned at 550°C for 1 h in a muffle furnace. They were then evaporated in an oven at 100 °C for 72 hours. After cooling down, membranes were weighted and the biomass concentration was determined in $g.L^{-1}$ as TSS. To determine the volatile fraction, the residue left in the membranes were ignited at 550 °C in a muffle furnace for 4 hours and then weighted to determine mass loss and ashes.

3.3.3 Biopolymer Quantification

Polyhydroxyalkanoates were quantified using gas chromatography according to Moita *et al.* [96]. Lyophilized biomass was incubated for 3.5 hours at 100°C with 1:1 solutions of chloroform with heptadecane as internal standard and a 20% acidic methanol solution. After the digestion step the organic phase of each sample was extracted with half a volume of water and injected into a gas chromatograph coupled to a FID (GC-FID, Bruker 400-GC). A Bruker BR-SWAX column (30 m × 0.25 mm × 0.25 µm) was used. Split injection at 280°C with a split ratio of 1:30 was used. The oven temperature program was the following: 40°C as initial temperature; then 20°C/min until 100°C; 3°C/min until 135°C; and finally 20°C/min until 220°C. The detector temperature was set at 230°C. Hydroxybutyrate and hydroxyvalerate concentrations were obtained using standards of a commercial P(HB-HV) polymer (88/12 %w/w, Aldrich).

Glucose biopolymer (GB) was extracted from lyophilized cells through acidic digestion (1 mL HCl 0.6 M, 2 h, 100°C). Digested samples were filtered before the liquid fraction being analyzed by HPLC using an Aminex HPX-87 H column (Bio-Rad Laboratories, CA, USA) at 60°C, and a Refractive Index detector (Merck, Germany), using H_2SO_4 0.01 N as eluent (0.5 mL/min).

3.3.4 Ammonium Quantification

The ammonium concentration was followed using a Thermo Scientific Ion Selective Electrode. To 1 mL of the samples were added 20 µL of Ionic Strength Adjuster (ISA). This solution which is composed of 5 M NaOH, 0.05 M EDTA, 10 % methanol and a blue dye, provides a constant background ionic strength and adjusts the solution pH. The calibration was done resorting to NH_4Cl standards (0-2.5 mM). Samples values were recorded in mV, after a measurement time of 5 minutes.

3.3.6 Chemical Oxygen Demand

Organic content of SBR1 samples were determined in terms of chemical oxygen demand according to the procedure described in Standard Methods [95]. A boiling mixture of chromic and sulfuric acids was used to oxidize the organic matter. To do so, two solutions, digestion solution (with the following composition: 10.216 $g.L^{-1}$ $K_2Cr_2O_7$, 16.7% (v/v) conc H_2SO_4, and 33.3 $g.L^{-1}$ $HgSO_4$), and sulfuric acid reagent (5.5 g Ag_2SO_4/kg H_2SO_4) were prepared and mixed in glass tubes in the proportion 3:7. Samples supernatant was diluted in order to achieve a COD value under 1 gCOD/L, and 2 mL were added to the mixture previously prepared. Subsequently, the sample tubes were placed on thermoreactor (Spectroquant® TR 620) at 150 ° C for 2h. After the digestion, the unreduced $K_2Cr_2O_7$ was measured with a colorimeter (Spectroquant® Picco Colorimeter COD/CSB). To determine COD values of samples, a glucose standard curve was performed (0-1.5 $g.L^{-1}$).

3.4 Microbial Community Analysis

3.4.1 Nile Blue Staining

2 drops of Nile blue solution (1 %w/v) were added to fresh bioreactor samples collected at the end of feast phase, and then heated at 55 °C, for 10 minutes. Samples were centrifuged for 2 min at 5000 rpm and washed with an 8 % acetic acid solution. A new centrifugation was made and the supernatant was discarded. The pellet was resuspended in 1xPBS buffer (Phosphate Buffered Saline) before observation under oil immersion at an epifluorescence microscope, Olympus BX51, equipped with an Olympus XM10 camera (Cell-F software). This technique was only applied to SBR2 samples.

3.4.2 FISH Analysis

Bioreactor samples taken with an interval of approximately 2 weeks were immediately fixed in formaldehyde after being collected and later tested for hybridization with several probes. Each specific probe was tested against the EUBmix and its content was visually estimated as a percentage of EUBmix hybridization.

a) Formaldehyde Fixation

The biomass was harvested by centrifugation and resuspended in 1xPBS. This washing step was performed 3 more times. Formaldehyde (4 %) was then added in a 1:1 ratio to the eppendorf with biomass resuspended in 1xPBS, and then incubated overnight at 4 ºC. Samples were centrifuged (5 min, 5000rpm) and the supernatant poured away. The *pellet* was resuspended in 1 volume of ice-cold 1xPBS and 1 volume of ice-cold 96 % (v/v) ethanol was added. The sample was stored at -20 °C.

b) FISH probes.

The FISH probes tested and respective nucleic acid sequence and modifications are listed in **Table 4**.

Table 4 *- FISH probes used in this study.*

Probe	Sequence 5'-3'	Target Organisms	Modification	Reference
ALF969	TGG TAA GGT TCT GCG CGT	*Alphaproteobacteria* (except of *Rickettsiales*)	Cy3	R. Lemaire and other unpublished results.
AMAR839	CTG CGA CAC CGA ACG GCA AGC C	*Amaricoccus* (except *A. tamworthensis*)	Cy3	[97]
ARC915	GTG CTC CCC CGC CAA TTC CT	*Archea* domain	Cy3	[98]
AZA483	GCC GTAV CTC TAG CCG TGC	*Azoarcus* sp.	Cy3	[99]
BET42a	GCC TTC CCA CTT CGT TT	*Betaproteobacteria*	Cy3	[100]
Cf319a	TGG TCC GTG TCT CAG TAC	*Flavobacteria, Bacteroidetes, Sphingobacteria*	Cy3	[101]
DECL585	ACG CCT GTC TTA CAA AAC CGC	Actinobacteria PAO	Cy3	---
DELTA495a Δ	AGT TAG CCG GTG CTT CCT	Most *Deltaproteobacteria* and most *Gemmatimonadetes*	Cy3	[102]
DELTA495b Δ	AGT TAG CCG GCG CTT CCT	Some *Deltaproteobacteria*	Cy3	

DELTA495c Δ	AAT TAG CCG GTG CTT CCT		Cy3	
EPSY549	CAG TGA TTC CGA GTA ACG	*Epsilonproteobacteria*	Cy3	[103]
EUB338 •	GCT GCC TCC CGT AGG AGT	Most Bacteria	FAM	[104]
EUB338-II •	GCA GCC ACC CGT AGG TGT	*Planctomycetales*	FAM	[105]
EUB338-III •	GCT GCC ACC CGT AGG TGT	*Verrucomicrobiales*	FAM	[105]
EUB IV •	GCA GCC TCC CGT AGG AGT	Bacterial lineages not covered by probes EUB338,EUB338-II e III	FAM	[106]
EUB338_V •	GCT GCC CCC CGT AGG AGT		FAM	[107]
GAM42a	GCC TTC CCA CAT CGT TT	*Gammaproteobacteria*	Cy3	[100]
Gnsb941	AAA CCA CAC GCT CCG CT	*Cloroflexi* (green nonsulfur bacteria)	Cy3	[108]
G_Rb	GTC AGT ATC GAG CCA GTG AG	*Rhodobacter, Roseobacter*	Cy3	---
Lgc354a ð	TGG AAG ATT CCC TAC TGC	*Firmicutes* (Gram + bacteria with low GC content)	Cy3	[109]
Lgc354b ð	CGG AAG ATT CCC TAC TGC		Cy3	
Lgc354c ð	CCG AAG ATT CCC TAC TGC		Cy3	
PAR1244	GGA TTA ACC CAC TGT CAC C	genus *Paracoccus*	Cy3	[110]
Pla46	GAC TTG CAT GCC TAA TCC	*Planctomycetales*	Cy3	[111]
SBR9-1a	AAG CGC AAG TTC CCA GGT TG	*Sphingomonas*-related organisms (Alpha-GAOs)	Cy3	[112]
TFO_DF218 ◊	GAA GCC TTT GCC CCT CAG	*Defluvicoccus*-related TFO (Alpha-GAOs)	Cy3	[113]
TFO_DF618 ◊	GCC TCA CTT GTC TAA CCG		Cy3	
THAU832	TGC ATT GCT GCT CCG AAC	*Thauera* spp.	Cy3	[114]
ZRA23a	CTG CCG TAC TCT AGT TAT	Most members of the *Zooglea* lineage, not *Z.resiniphila*	Cy3	[115]

Δ Used as DELTAmix; • Used as EUBmix; ð Used as Lgcmix; ◊ Used as TFOmix

c) Hybridization on slides.

Sludge samples fixed in 4 % formaldehyde were placed in individual wells of a Teflon coated slide, (2 - 15 µL, amount depending on the cell concentration) and dried for about 10 min at 46 °C. The slides were then dehydrated in an increasing ethanol series (3 min each in 50, 80 and 100 % ethanol). 10 µL of hybridization buffer (previously prepared according to stringency of the probes used) was dropped onto the wells, and 0.5 µL of each probe was added (concentration 30 ng/ µL for Cy3 labeled probes, and 50 ng/µL for 6-FAM labeled probes). The hybridization tube (50 mL Falcon tube) was prepared by folding a piece of tissue, putting it into the tube and pouring the rest of the hybridization buffer onto the

tissue. Slides were immediately transferred onto the hybridization tube and incubated in the hybridization oven (46 °C) for at least 1.5 hours. Hybridization buffer was rinsed from the slide by incubating the slides in the washing buffer for 10 min in a water bath (48 °C). Washing buffer was removed with distilled water and slides were air-dried. The slide was embedded with Vectashield mounting media and putted a cover slip onto the slide. The cover slip was sealed and the slides were kept at -20 °C. The slides were observed under oil immersion at 1000x magnification resorting to an epifluorescence microscope, Olympus BX51, equipped with an Olympus XM10 camera (Cell-F software).

3.4.3 DGGE

a) DNA extraction for 16S ribosomal DNA analysis.

DNA was extracted from samples taken using the Ultraclean Microbial DNA isolation KIT. The efficiency of extraction was determined by direct nanodrop measure, where DNA concentration, 260/280 and 260/230 ratios were determined.

b) Primers and PCR amplification.

From DNA extracted, 16S rDNA fragments were amplified with the primers presented in **Table 5**, which are specific for universally conserved bacterial 16 rDNA sequences. PCRs were performed with a mixture of 5x RANGER Reaction Buffer 10 µL, which contains dNTPs, $MgCl_2$ (1.5mM final concentration) and enhancers; 50 pmol of each primer, 5 µL of template and water up to 50 µL. The protocol was for a standard 50 µL amplification of 10kb fragments.

The PCR reaction was programed to do an initial denaturation at 95 °C for 1 minute and then repeat for 35 cycles the following steps:

- 10 seconds denaturing step at 95°C.
- Primer annealing was performed for 30 seconds at 58 °C
- Primer extension was performed at 68 °C for 1 minute.

Finally, an extension step of 7 minutes at 68 °C was performed

Table 5 *- Primer sequences and positions*

Primer	Position	Target	Sequence
968f-GC	968-984	Bacteria, regions V6-V8	5'-GC-clamp-AACGCGAAGAACCTTAC 3'
1401r	1385-1401	Bacteria, regions V6-V8	5' –CGGTGTGTACAAGACCC- 3'
GC-clamp			5'-CGCCCGGGGCGCGCCCCGGGCGGGGCGGGGGCACGGGGGG-3'

After the last cycle, the samples were cooled down until 4 ºC and an agarose (2 %) gel electrophoresis was performed to check if there was amplification.

c) DGGE analysis.

PCR products were loaded onto a 8 % (w/v) polyacrylamide gel in 1/2X buffer (40 mM Tris, 20 mM acetic acid and 1 mM EDTA at pH 8). Initially the 8% (w/v) polyacrylamide gels were made with a denaturing gradient ranging from 20-80% and then the gradient was narrowed to 45-55%. Denaturant (100%) contained 7M urea and 40% formamide. The electrophoresis was run at 60 °C, for 10 minutes at 20 V, and subsequently for 16 hours at 60 V. After electrophoresis, gels were stained for 30 minutes with SYBR Safe DNA gel stain and then observed and photographed in Safe Imager 2.0 Blue-Light Transilluminator.

Digitized DGGE images were analyzed with GelCompar II software. Lanes and reference bands were applied to each gel image by the software, with manual fine-tuning of the band designations. Subsequently, similarities between the densitometric curves of the band patterns were calculated based on the Dice coefficient and DGGE patterns were clustered based on the unweighted pair group method with arithmetic mean (UPGMA) clustering algorithm.

3.5 Calculations

The sludge PHA and GB content was calculated as a percentage of TSS on a mass basis:

$$\%PHA = \frac{gHA}{g\ TSS} \times 100 \quad , \quad \%GB = \frac{g\ glucose}{g\ TSS} \times 100$$

Active biomass (X) was obtained by subtracting the storage products from VSS as:

$$X = VSS - PHA - GB \text{ (in g/L)}$$

It was assumed that all the ammonia consumed was used for growth since using thiourea inhibited nitrification. Active biomass elemental composition was represented by the molecular formula $C_5H_7NO_2$ [116]. Substrates were converted to $gCOD.L^{-1}$ using the respective chemical oxidation equation and active biomass was converted into COD according to a conversion factor of 1.42 mgCOD/mg biomass [8].

The specific substrate uptake rates (xylose specific consumption rate (qs_{Xyl}), acetate specific consumption rate (qs_{Acet}), glycerol specific consumption rate ($-qs_{Gly}$), methanol specific consumption rate ($-qs_{Meth}$)) and biopolymers production rates (PHA specific production rate (qs_{HA}), glucose biopolymer specific production rate (qs_{GB})) were determined by adjusting a linear function to the experimental data for each variable concentration divided by the biomass concentration at that point along time, and calculating the first derivative at time zero.

The HB production yield ($Y_{HB/S}$), GB production yield ($Y_{GB/S}$) and biomass production yield ($Y_{X/S}$) on substrate were calculated by dividing the amount of each parameter by the total amount of substrate consumed (S). For SBR1, S was calculated as the sum of acetate and xylose concentrations while for SBR2 glycerol and methanol were accounted to S determination.

4

RESULTS AND DISCUSSION

4.1 HSSL fed reactor (SBR1)

With the purpose to achieve a homogenous culture with a high PHA-accumulating capacity adapted to a complex substrate such as HSSL, a sequencing batch reactor (SBR1) fed with this feedstock (OLR=17.3 gCOD.L-$^{-1}$.day^{-1}) was operated continuously for 97 days under ADF conditions. Along the operational period several parameters were monitored: COD consumption because HSSL is a complex mixture of carbon sources; the two main carbon sources, acetic acid and xylose; ammonia; PHA and biomass production. These parameters allowed to evaluate the acclimatization of the MMC to the operational conditions and its production capacity.

4.1.1 PHA-accumulating culture enrichment

a) SBR cycle

As mentioned before, SBR1 was operated continuously in ADF conditions for 97 days during which successive 8h cycles were repeated. Along that period, SBR1 was characterized through monitorization of individual cycles on a weekly basis that allowed accessing the system performance along time. **Figure 15** shows the typical behavior of the culture during a representative SBR cycle, obtained on the 51st day of operation.

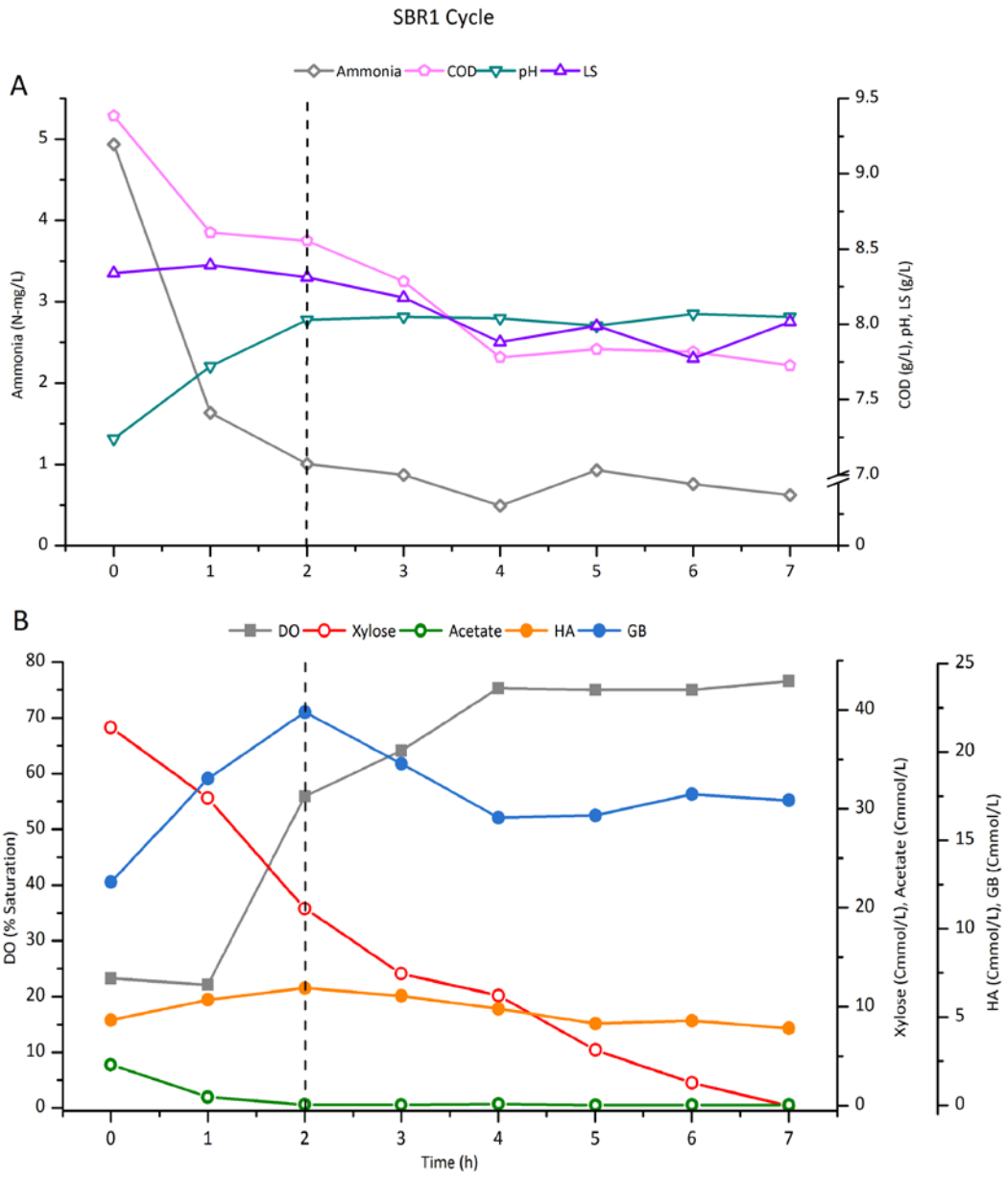


Figure 15 *- SBR1 cycle at 51st day of operation.*

Since the system did not have pH control, the pH profile in the reactor varied along the cycle. At the beginning (t=0h), a pH of 7.24 was observed and it resulted from the addition of fresh medium. Then, as expected, pH increased until t=2h (pH=8.03), the time at which acetate was completely depleted. After t=2h, pH value remained stable until the end of the cycle. The lower pH variation observed when comparing to the pH variation between 8.0 and 10.0 observed by Serafim *et al.* [64], in a SBR fed with acetic acid without

pH control, can be, as hypothesized by Queirós *et al.* [88], the result of a possible buffer behavior of HSSL.

HSSL mainly contains three different carbon sources: lignosulphonates, xylose and acetate. LS consumption exhibited a variable behavior between cycles and although a slight consumption seemed to occur along the cycle, there was not a clear tendency and consumption rates could not be determined **(Figure 15A)**. Even though consumption was not significant, the microbial community seemed to tolerate the presence of such components, thus not showing inhibitory effect. Regarding xylose and acetate, they were both consumed and depleted along the cycle, although at different rates. While acetate was totally consumed until t=2h at 1.09 Cmmol/gX.h, xylose, due to its higher concentration, was only exhausted at the end of the cycle. Xylose uptake rate was not constant through the whole cycle: it was higher until 1h after acetate exhaustion (2.86 Cmmol/gX.h), decreasing to 1.17 Cmmol/gX.h afterwards. Similar profiles for carbon sources were observed by Moita *et al.* [66] when using crude glycerol to select a PHA-storing MMC.

The evolution of external carbon sources concentration is usually reported to be correlated with DO concentration in many ADF systems found in the literature. The time of the exhaustion of the carbon source corresponds to an abrupt increase of DO in the medium, but in this case, DO only reacted to acetate. Since the different carbon sources present were consumed in different ways, the definition of the feast and famine phase cannot be the same for all the different microorganisms in the culture. In the case of the cycle of **Figure 15**, the feast phase had the duration between 1 to 2 hours (more frequent samples should have been taken in order to determine the exact moment of acetate exhaustion), and the famine phase between 5 to 6 hours considering only acetic acid and oxygen consumption. Nevertheless, this feast/famine regime was applicable to organisms that consumed acetic acid, not being, however, a true feast/famine for the organisms that consumed other carbon sources present in HSSL like xylose. Other than acetate, the large variety of carbon sources that exist in the liquor remained along the entire cycle as shown by COD, allowing diverse microbial populations to co-exist in the system. Consequently, populations without the ability to store polymers were able to grow and persisted in the

SBR throughout the consumption of such carbon sources. In fact, the sugar fraction, namely xylose, existent in the HSSL was continuously consumed through the cycle and did not seem to contribute for the PHA production. Instead, this may be one of the possible substrates used by the microbial population unable to accumulate polymers inhibiting the selection purpose of the ADF conditions by decreasing the selective pressure imposed. Moita *et al.* [96] observed the same culture behavior using bio-oil resultant from pyrolysis process which contained organic acid and sugar fractions.

Regarding the PHA storage, accumulation occurred at a specific rate of 0.0061 gCOD HA/gCOD X.h until reaching the maximum content of PHA (3.66% gHA/gTSS) when the acetate was fully consumed (at the end of the feast phase). The specific PHA accumulation rate obtained was below the range reported in the literature (0.0082–0.42 gCOD HA/gCOD X.h) as well as the storage yield of $Y_{PHA/S}$=0.075 gCOD HA/g SCOD (0.24–1 gCOD HA/gSCOD) [52, 66]. This was expected since the xylose consumption was taken into account to the PHA yield calculation and probably did not contribute to its accumulation. In order to clarify the contribution of each carbon source to polymer accumulation, batch tests should have been done with each substrate individually.

GB was also produced during the feast phase along with PHA. Comparing the specific production rates of both biopolymers, GB synthesis (1.56 Cmmol GB/gX.h) was more than five times faster than PHA. The maximum GB content coincided with the PHA maximum although being significantly higher (17.11% gGluc/gTSS). Also GB storage yield (0.43 Cmmol GB/Cmmol S) was much higher than the PHA storage yield. These results were consistent with the findings by Dircks *et al.* which showed that glycogen storage was faster than PHA production and more efficient in terms of ATP than PHB [117]. After reaching their maximum value, the amount of PHA and GB decreased during the famine phase due to its consumption by bacteria for their maintenance and growth, although xylose and LS were still present and being consumed.

The data presented above and in **Figure 16** suggest that the system was more specialized in GB accumulation rather than PHA. In order to change that, and utilize the available carbon sources more efficiently to produce PHA, it would be advised the introduction of a pre-fermentation step in the process to convert sugars into VFAs through acidogenic fermentation. This strategy would not only decrease the production of glycogen

but also provide a more suitable substrate for PHA production. Also the imposed operational conditions may not be the most appropriate. The use of longer cycle duration should be considered in order to increase the F/F ratio of the system and thus increase the selective pressure on the microbial community.

b) SBR performance

Generally feast to famine ratio (F/F ratio) is considered a good indicator to access the stability of the SBR [89]. This ratio is determined using DO in the medium as an indicator, since usually, when the exhaustion of the carbon source occurs, an abrupt increase on this value is verified. That allows identifying the transition between the two phases and determining their duration in an easy and practical way. Since on-line data acquisition of DO concentration values were not available on this system, F/F ratio values were determined based on the DO values registered at the time of samples collection in the days of cycle analysis. The F/F ratio evolution along operational period of the SBR1 is shown in Figure 16. It is possible to observe that after an initial adaptation period where F/F ratio remained high at 0.5, it dropped to 0.25 after the 21st day of operation, where it remained stable until the end of operation.

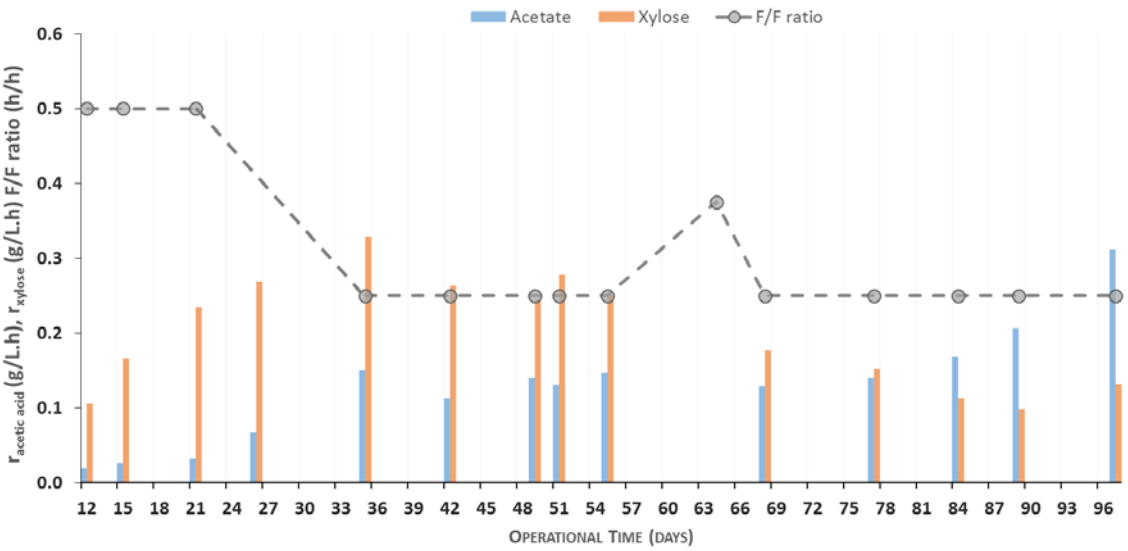


Figure 16 *- Evolution of F/F ratio along the operational period of SBR1. Uptake rate variation of the most important carbon sources (xylose and acetic acid) along the operational period of SBR1.*

The F/F ratio is an important parameter to take in account in this type of systems and it can be varied in an SBR by changing the OLR. Low OLR is generally used to operate the selection stage [55, 64, 118], resulting in low F/F ratios and, consequently, favoring a selective pressure for PHA-storing microorganisms [61]. Different studies [56, 70] showed this correlation, indicating F/F ratio values up to 0.26 responsible for high storage response while for higher ratios, a very unstable storage or, predominantly, growth responses were obtained. The stable F/F ratio value of 0.25 obtained in SBR1, although near the limit value, is comparable to the values indicated in the literature associated with a predominantly storage response.

However, it is important to bear in mind that F/F ratio values calculated in this system were not totally accurate since samples were collected at 1h intervals, which did not necessarily match with the transition between feast and famine phases.

As such, other parameters were used to access reactor performance such as consumption rates and production yields. Evolution of xylose and acetic acid uptake rates along the operational period is represented in **Figure 16.** In HSSL composition, (**Table 3**) other carbon compounds present can act as potential substrates for the MMC. However, regarding LS, its determination did not show a clear tendency along SBR cycles thus not revealing a significant consumption. The evolution of the remaining sugars of HSSL (mannose, arabinose, galactose, glucose rhamnose, fucose) was not followed because their concentration in the medium after HSSL dilution was under the detection limit of the HPLC column.

The results of substrates uptake rates showed that between these two compounds, xylose was preferably consumed until 77th day of operation and its consumption rate increased from the beginning of operation until the maximum value at day 35 (0.33 $g.L^{-1}.h^{-1}$) and after that a consistent decrease was verified until reaching the value of 0.13 $g.L^{-1}.h^{-1}$ at the end of operation. On the other side, acetic acid, after starting with a low consumption rate (0.019 $g.L^{-1}.h^{-1}$) evolved along the operational period with constant growth even surpassing xylose as preferable substrate after 77th day and reaching a maximum value of 0.31 $g.L^{-1}.h^{-1}$ at 97th day.

MMC are known to preferentially consume VFAs for PHA production and as reported by many authors, they do not accumulate carbohydrates (such as xylose and glucose) as PHAs [79, 117]. Instead, carbohydrate consumption are associated with glycogen storage since it is more energetic favorable than storage as PHA in MMC [117]. Indeed, it has been observed that in presence of both carbohydrates and acetic acid, the latter is stored as PHB whereas the former compounds, such as glucose or starch, are stored as glycogen [79, 119].

With this in mind, and the increasing acetic acid consumption rate along time, it is believed that culture enrichment occurred for organisms that preferably consume acetic acid. Still, data also shows that the system was unable to reach a pseudo steady state. As shown also by **Figures 16-17**, constant kinetic and stoichiometric parameters were not observed in spite of an operational period of 97 days (19.4 SRT). Although some reported values range between 30 and 167 days for the selection reactors, there are not reference of stabilization periods for systems using HSSL as feedstock in the literature, which leads us to believe that a longer operational period was necessary to achieve a stable culture [8, 56, 120].

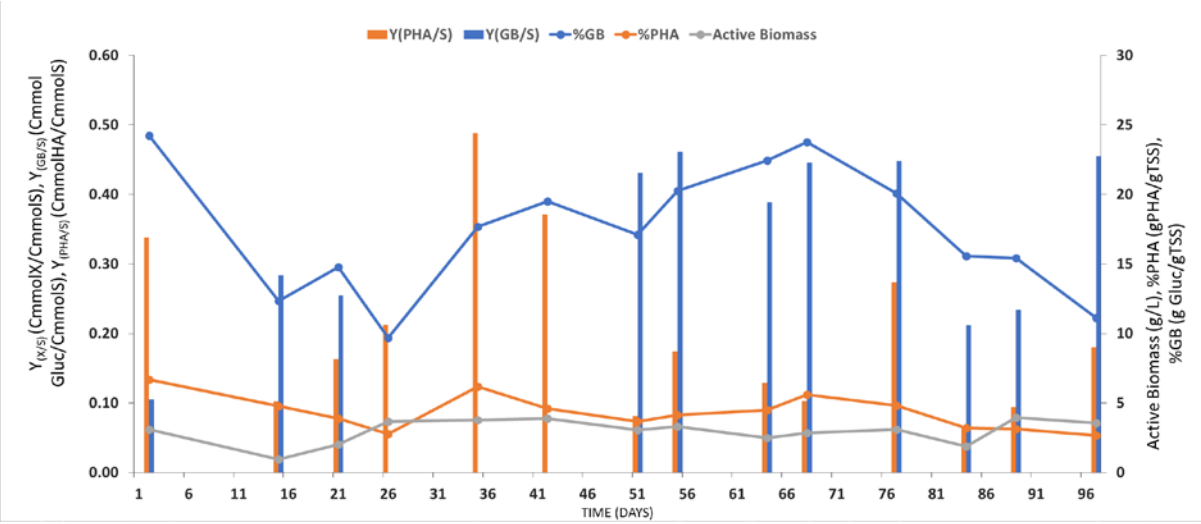


Figure 17 *- Evolution of several yields and accumulation percentages of polymers along the operational period.*

Besides insufficient time of operation, possible microbial inhibitors present in HSSL may be another explanation for reactor performance instability. Using HSSL as feedstock for PHA production by MMC, *Queirós et al.* [88] reported similar culture instability. As reviewed by *Pereira et al.* [86], HSSL microbial inhibitors can have a synergistic inhibitory effect on microbial growth and metabolic pathways. Compounds such as gallic acid,

pyrogallol and furfural are known to have a toxic effect on bacteria which can affect the production of active biomass and PHA [86, 93].

Nevertheless, the obtained results show that, despite the presence of these inhibitors, a PHA-storing MMC with tolerance to these compounds was able to adapt to the imposed conditions on the SBR and to use at least some of the components of HSSL as substrate. As seen in **Figure 17,** this population was able to maintain an average value of active biomass of 2.85 ± 0.88 $g.L^{-1}$ and a relatively low but stable PHA content along time of operation of 4.31 ± 1.18%. The highest PHA content obtained in all acclimatization process was 6.66% right on the 2nd day but the highest PHA storage yield occurred on day 35 (0.45 gCOD HA/g SCOD). The PHA content was slightly lower than the range observed for the selection step of other reported works [8, 78, 121]. However, the highest storage yield is on the range of the values of similar reported works obtained in the accumulation assays (0.24–1 gCOD HA/g COD) [66].

As expected, considering the high sugar content in HSSL, a significant higher, but also more variable, glycogen content was obtained (18.32 ± 4.14 %), reaching the maximum value of 24.21% at the beginning of operation. As a result, when comparing to PHA production yield, a higher glycogen accumulation yield was generally obtained.

Since biomass growth and polymer accumulation usually compete for the carbon sources it would be expected to establish a relationship between them [52]. However, according to experimental data, there was not a clear tendency between such parameters. While active biomass remains fairly stable after day 26, polymer accumulation yields had different tendencies: PHA accumulation yield seemed to decrease but with an irregular behavior while glycogen accumulation had an increase tendency but also in a non-consistent way. This might be due to the complexity of the raw material used as substrate and the different bacterial groups present in the culture, which consumed the different carbon sources without competition [73]. Nevertheless, although analysis of daily cycles demonstrated a culture preference for substrate storage as reserve polymers, substantial enrichment of a PHA accumulating culture was not verified in comparison to the initial MMC.

Stoichiometric and kinetics parameters were calculated for monitored daily cycles and average values are presented in **Table 6**.

Table 6 *- Kinetic and stoichiometric parameters obtained for the SBR1.*

Assay	$-q_{Acet}$	$-q_{Xyl}$	q_{HA}	q_{GB}	$\%HA_{max}$	$\%GB_{max}$	X	$Y_{HA/S}$	$Y_{GB/S}$
Average daily cycles	1.08 (0.50)	2.65 (1.74)	0.72 (0.53)	2.10 (1.16)	4.31 (1.18)	18.32 (4.14)	2.85 (0.88)	0.20 (0.13)	0.34 (0.12)
Cycle day 51	1.09	2.86	0.29	1.56	3.66	17.11	3.04	0.08	0.43
Accumulation									
1º pulse	0.77	3.15	0.47	nd	3.11	nd	3.19	0.11	nd
2º pulse	0.71	2.54	0.34	nd	3.73	nd		0.10	nd
3º pulse	0.73	4.39	0.43	nd	4.65	nd		0.14	nd

(standard deviation); (nd) - not determined.
q_{Acet} (Cmmol Acet/Cmmol X.h); q_{Xyl} (Cmmol Xyl/Cmmol X.h); q_{HA} (Cmmol HA/Cmmol X.h); q_{GB} (Cmmol Gluc/Cmmol X.h).
$\%HA_{max}$ (% gHA/g TSS); $\%GB_{max}$ (% g Gluc/g TSS).
X ($g.L^{-1}$); $Y_{HA/S}$ (Cmmol HA/Cmmol S); $Y_{GB/S}$ (Cmmol Gluc/Cmmol S).

4.1.2 Accumulation assay

With the objective of determine the maximum PHA storage capacity of the selected culture, an accumulation test was performed at 58th day regarding a multiple pulse addition of HSSL in order to overcome potential inhibition promoted by substrate. The obtained results are presented in **Figure 18**.

PHAs were produced at a similar rate along the three pulses reaching, an average specific PHA accumulation rate of 0.42±0.065 Cmmol/gX.h and a storage yield of 0.12 ± 0.021 Cmmol HA/Cmmol S. Kinetic parameters for each pulse are listed in **Table 6**.

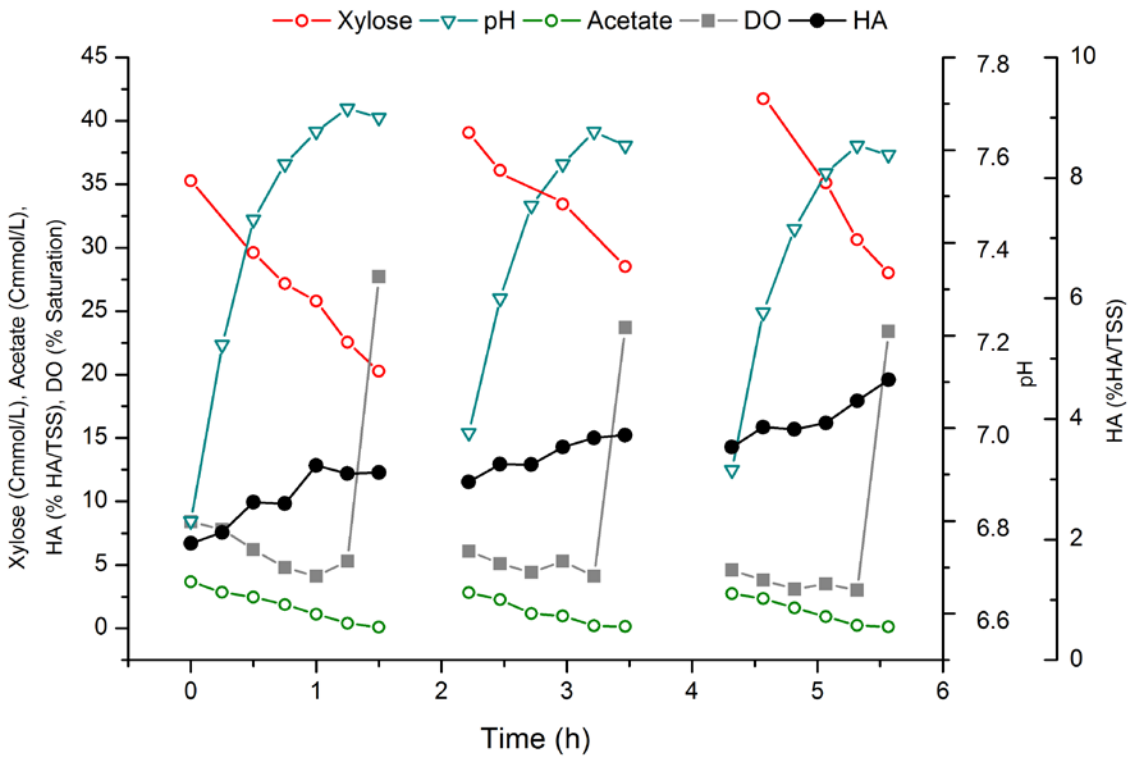


Figure 18 *- PHA accumulation assay performed at 58th day of operation.*

The maximum PHA content achieved of 4.6% at the end of the third pulse was substantially lower than other works that used real complex substrates, which ranged from 25 to 77% [66, 67, 88]. As discussed before, the low PHA content of the culture may be due to the lower VFAs content of the HSSL, which are the main precursors to produce PHA from MMC and due to the presence of populations unable to accumulate PHAs that can co-exist in the system and may be responsible for the consumption of the sugar-based compounds registered during the entire assay. Besides that, the strategy used may not be the most adequate. In this test, only three pulses of substrate were added. Considering that in the end of the third pulse the culture was still able to accumulate PHAs and consume the substrates without any sign of inhibition, more pulses should have been added in order to improve the PHA content in cells. Also future accumulation tests should be performed with limiting conditions of ammonium and oxygen in order to channel the carbon consumed to polymer accumulation and minimize biomass growth.

4.1.3 Microbial Community Analysis

With the purpose of fully understand the microbial processes taking place in the bioreactor, the study of the microbial community and its evolution is required in order to determine which organisms are present and doing what. As such, the bacterial community selected in the SBR1 was taxonomically characterized through the operational time by FISH analysis. After this approach, the microbial speciation process occurring in the SBR was also investigated resorting to a culture independent molecular method, DGGE.

a) FISH Analysis

The adopted strategy for community analysis along reactor operation through FISH technique started at the higher taxonomic levels testing the culture with specific probes for the *Archaea* and *Bacteria* domains. The screening for the presence of *Archaea* did not show hybridization in any of the analyzed samples while Bacteria was abundantly present as seen in **Figure 19**.

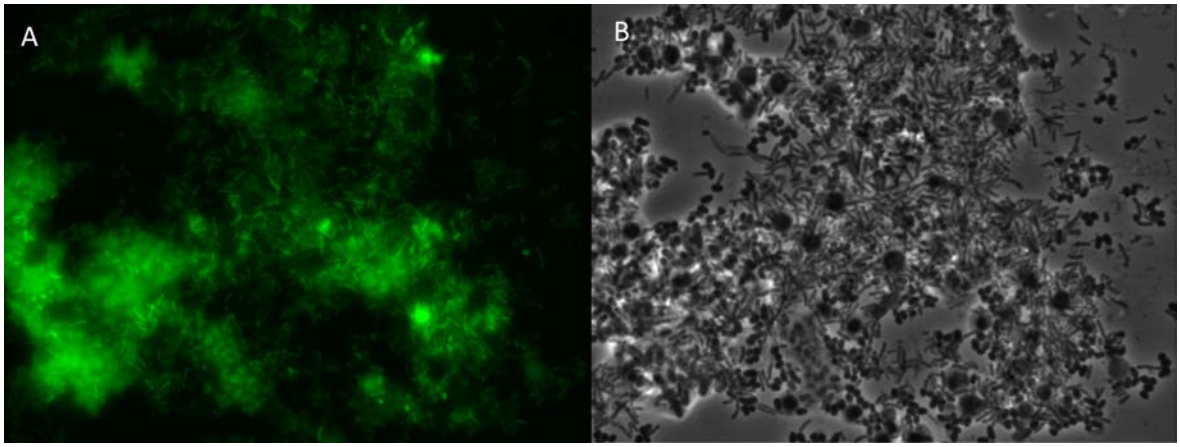


Figure 19 *- Microscope images of the microbial culture. EUBmix probe (A); phase contrast image (B). Magnification 1000×.*

Next, a successively refinement of the taxonomic composition belonging to bacteria domain until the *genus* level was performed. The result of this analysis allowed to monitor the evolution of the identified taxonomical groups bacterial in the microbial community throughout the entire reactor operation. In **Figure 20** it is shown the evolution of the identified classes of organisms present in the microbial culture.

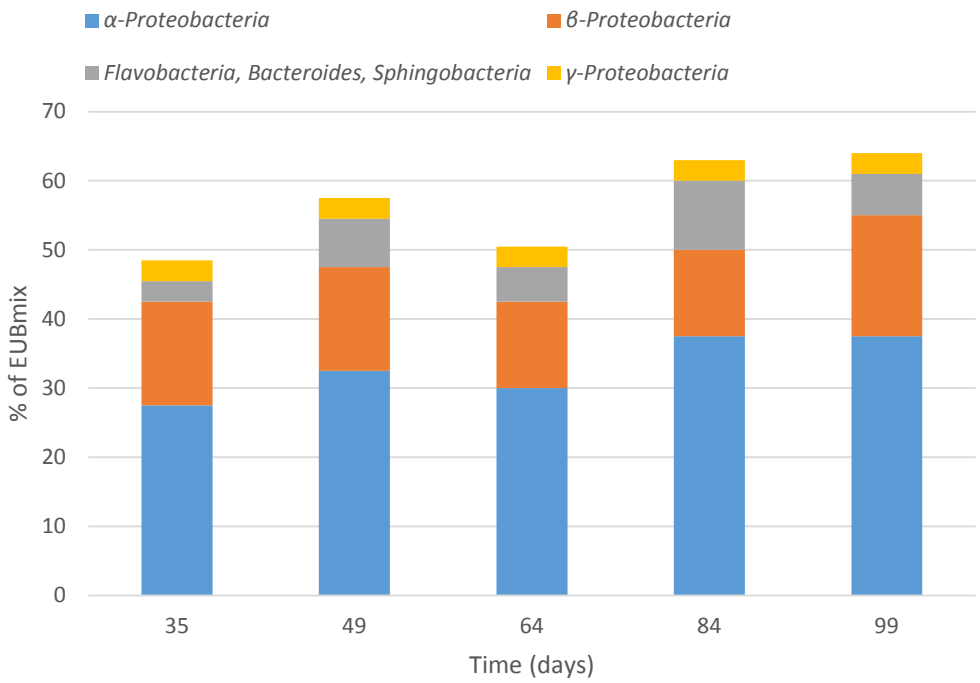


Figure 20 *- Bacterial community evolution along the operational period of SBR1.*

The classes identified in the selected culture comprised *Alphaproteobacteria*, *Betaproteobacteria*, *Gammaproteobacteria* and *Flavobacteria, Bacteroides, Sphingobacteria*. Their sum only accounted for 48-69% of EUBmix hybridization cells, which means that other classes not targeted are also present. Observing the data, the most abundant class of organisms since 35th day of operation was *Alphaproteobacteria* that increased from 27% to 37%. The next most representative class was *Betaproteobacteria* that started at 15% and remained fairly stable until 99th day with 17%. The percentage of hybridization of the probe specific for the group of classes *Flavobacteria, Bacteroides* and *Sphingobacteria* revealed some fluctuations but without surpassing 10% content. Regarding *Gammaproteobacteria,* its content remained unchanged at 3% of the total EUBmix hybridization. Several PHA accumulating microbial communities characterized in the literature report *Betaproteobacteria* as the major class present [8, 75, 77]. However, using the same feedstock, Queirós *et al.* [88] described a similar community composition although *Deltaproteobacteria* and *Actinobacteria* were also found to be present. The *Alphaproteobacteria* progressive increase match the acetate uptake rate increase seen in

Figure 16 thus being possibly related. Correlation between microbial classes composition and PHA accumulation yield or PHA content does not appear to be present.

The *Alphaproteobacteria*, and *Betaproteobacteria* classes, as the most representative of the microbial culture were further identified at the *genus* level. From the several probes tested, which are specific for several *genera* usually reported to be present in PHA accumulating microbial cultures, only two *genera* of *Alphaproteobacteria* were found: *Paracoccus* and *Rhodobacter.* Their content evolution is shown in **Figure 21**.

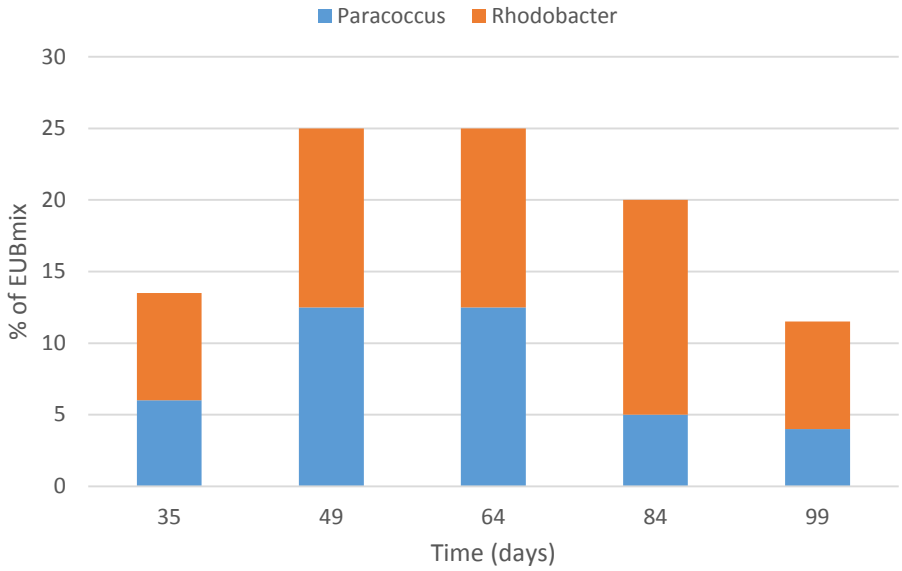


Figure 21 *- Evolution of Paracoccus and Rhodobacter content during the reactor operation.*

Between these two genera only *Paracoccus* was identified by Queirós *et al. [88].* However, several studies reported *Rhodobacter* as being present in selected PHA accumulating cultures and this genus is known to be able to produce PHAs [122, 123].

It should be noted that several specific probes that hybridize with known PHA accumulating organisms (*Thauera, Amaricocus, Azoarcus* and *Zooglea* genus) were also tested with a negative result obtained.

The microscopic images with positive result for the probes tested in sample of 99th day of operation are analyzed below (**Figures 22-27**). All the other probes tested were negative.

In **Figure 22,** is shown the result obtained for the *Alphaproteobacteria* targeted probe (Alf968 probe). Cells where hybridization occurred are shown in, where the green cells represent biomass that bound to EUBmix probe for Bacteria Domain. As shown in the picture, it is clear that the predominant morphotype associated with *Alphaproteobacteria* are *bacilli* but also *coccobacilli* seemed to be present although at a fewer extent.

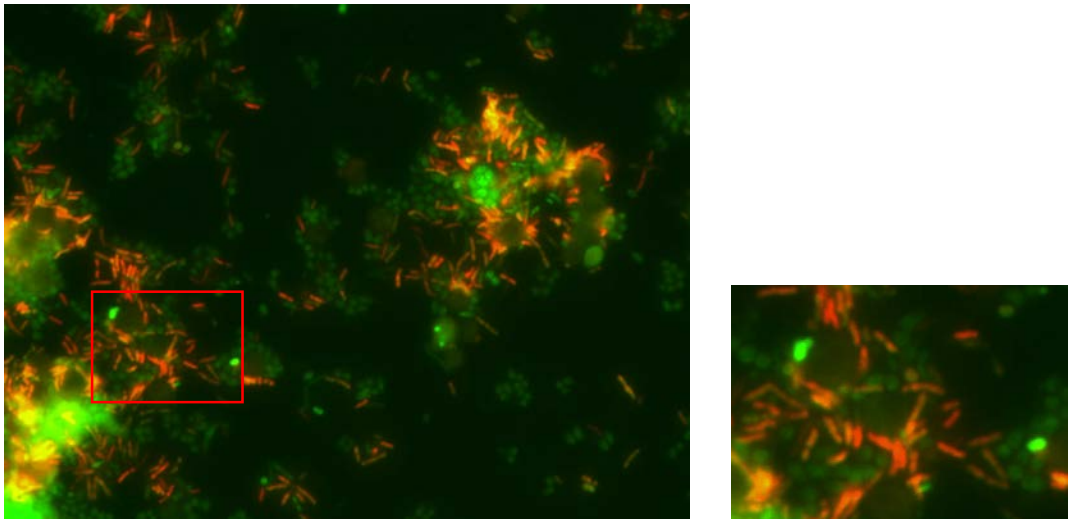


***Figure 22** - Overlap of FISH pictures and amplification of detail. Orange cells were hybridized with Alf 968 probe and green cells represent the biomass that hybridized with EUBmix probe. Probes applied to sample correspondent to the 99th day of operation. Magnification of 1000x.*

Relatively to the other major bacterial class present, *Betaproteobacteria*, they had a very characteristic morphology. Cells that hybridized with BET42a probe were always presented in cocci aggregates as seen in **Figure 23**. As far as we know, other study of PHA accumulating culture characterization had not reported a similar morphology.

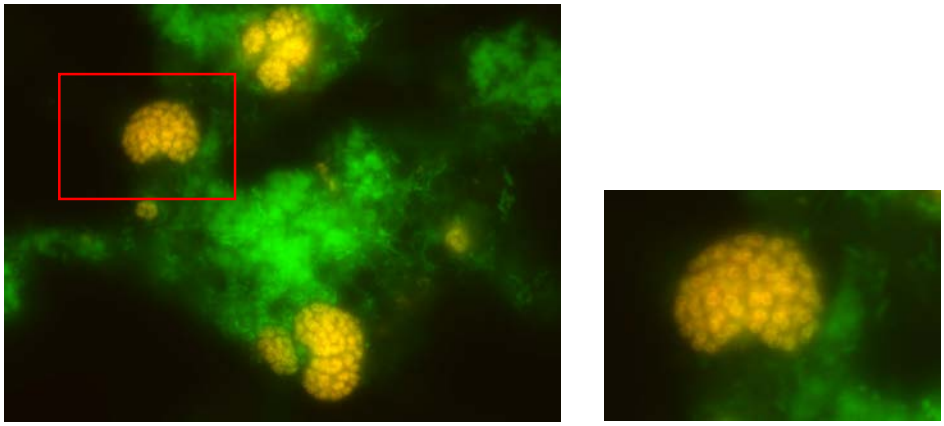


Figure 23 *- Overlap of FISH pictures and amplification of detail. Orange cells were hybridized with BET42a probe and green cells represent the biomass that hybridized with EUBmix probe. Probes applied to sample correspondent to the 99th day of operation. Magnification of 1000x.*

Gammaproteobacteria probe hybridization revealed the presence of bacteria belonging to this *genus* with coccobacilli morphology as seen in **Figure 24**.

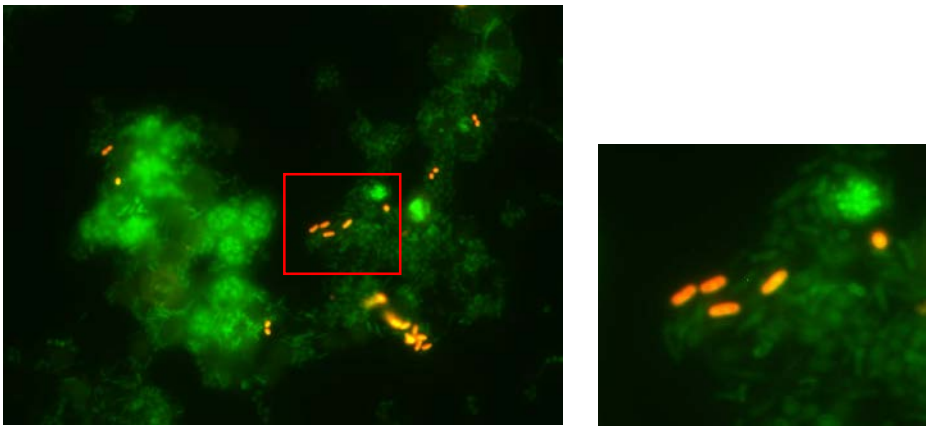


Figure 24 *- Overlap of FISH pictures and amplification of detail. Orange cells were hybridized with GAM42a probe and green cells represent the biomass that hybridized with EUBmix probe. Probes applied to sample correspondent to the 99th day of operation. Magnification of 1000x.*

Another probe applied, Cf319a, allowed the identification organisms belonging to the *Bacteroidetes* phylum, specifically to the *Flavobacteria*, *Bacteroides*, *Sphingobacteria genera*. This probe was applied because these bacteria are well described as environmental bacteria, and therefore very susceptible to being present in activated sludge (**Figure 25).** Although it is not very clear, it seemed that the morphologies present were cocci and bacilli.

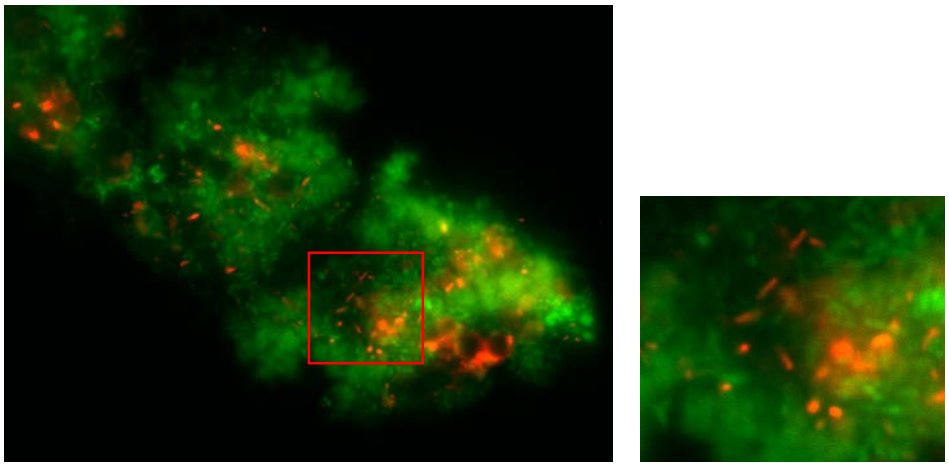


Figure 25 *- Overlap of FISH pictures and amplification of detail. Orange cells were hybridized with Cf319a probe and green cells represent the biomass that hybridized with EUBmix probe. Probes applied to sample correspondent to the 99th day of operation. Magnification of 1000x.*

At the genus level only *Paracoccus* and *Rhodobacter* belonging to *Alphaproteobacteria* had positive results. In both cases (**Figure 26-27**), a coccobacilli morphology was verified.

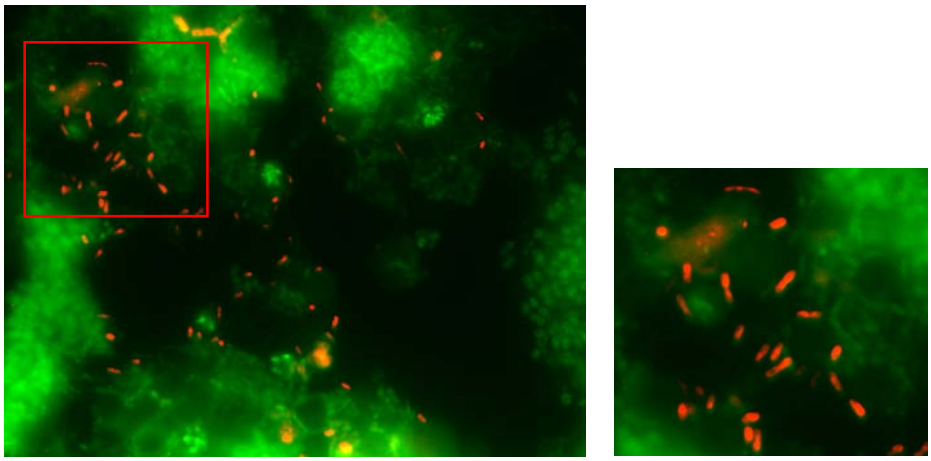


Figure 26 *- Overlap of FISH pictures and amplification of detail. Orange cells were hybridized with PAR1244 probe and green cells represent the biomass that hybridized with EUBmix probe. Probes applied to sample correspondent to the 99th day of operation. Magnification of 1000x.*

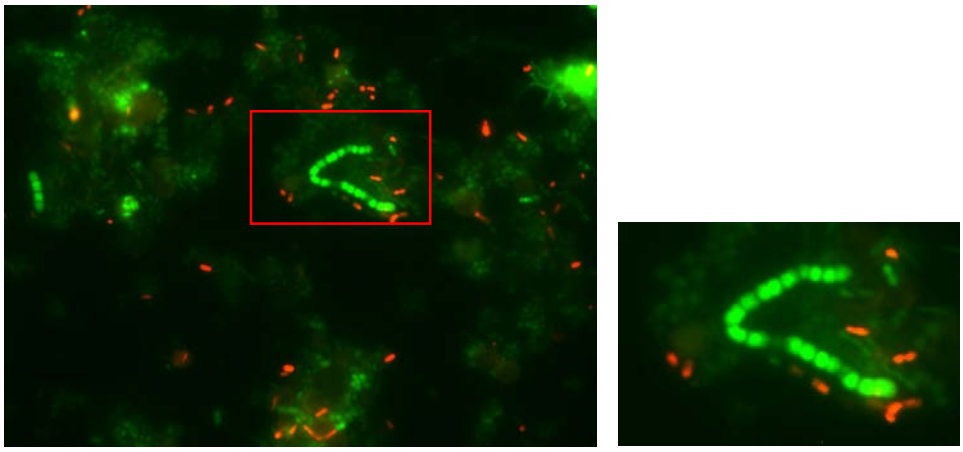


Figure 27 *- Overlap of FISH pictures and amplification of detail. Orange cells were hybridized with G_Rb probe and green cells represent the biomass that hybridized with EUBmix probe. Probes applied to sample correspondent to the 99th day of operation. Magnification of 1000x.*

b) DGGE Analysis

In order to know how the operational conditions affected the bacterial richness and dynamics in different stages of the process, PCR-DGGE technique was applied. The numbers of PCR products, reflected as bands migrating in each DGGE gel represents the number of 16S rRNA gene sequence similarity groups or operational taxonomic units, which usually are called species for simplicity.

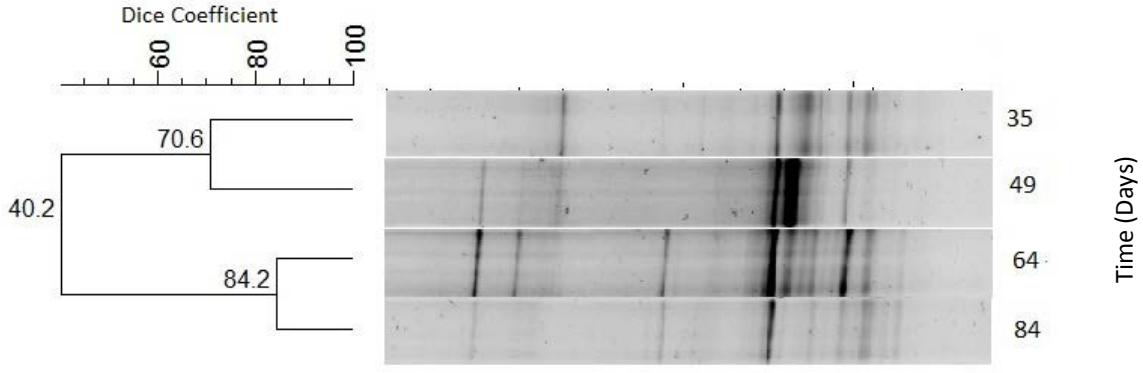


Figure 28 *- DGGE profile of the bacterial community Structure throughout the SBR1 operation and respective cluster analysis tree constructed using the Unweighted Pair Group Method with Arithmetic Mean (UPGMA) clustering algorithm.*

As shown in **Figure 28**, it is possible to observe the existence of an increasingly heterogeneous microbial community until day 64, with some evidence for the presence of some dominant species (especially in samples 49 and 64) represented by the most intense bands.

During the SBR operation a considerable shift in the species composition of the microbial population occurred between day 49 and 64 as confirmed by the value of the similarity coefficient between these two samples (40.2). Between samples 64 and 84, there is a high similarity although microbial diversity seems to decrease in the last sample. These results confirm, on a molecular base, the ineffectiveness of imposed conditions in progressively selecting a predominant restricted microbial population from the initial inoculum. Therefore being in agreement with the reactor performance results, where a specialized PHA storage community was not achieved. The sequencing of the bands in DGGE profile would allow identifying the species present in the culture and therefore accessing their capability of PHA accumulation.

4.2 Crude Glycerol fed reactor (SBR2)

SBR2 was started under ADF condition to select a culture able to accumulate PHA using CG as feedstock (OLR=30 CmM.d^{-1}) and operated for a period of 129 days. Since the bioreactor was inoculated with a previously PHA-accumulating culture selected with CG it was expected that inoculum adaptation to substrates would be faster and that it would minimize the fact that glycerol and methanol are not preferred substrates for PHA production like VFAs [66].

Reactor monitorization included the quantification of main carbon sources (glycerol and methanol) and the polymers produced (PHA and GB), as well as ammonia consumption and biomass production. Such parameters allowed characterizing the selected culture and evaluating their accumulation capacity.

4.2.1 PHA-accumulating culture enrichment

a) SBR cycle

SBR2 performance was determined based on the behavior of the culture in cycles analyzed in a weekly basis. A typical 24h cycle obtained at day 86, under steady state operational conditions, is show in **Figure 29.** The results shown are regarding the first 8 h only, since significant changes did not occur in the remaining cycle time where culture undergoes a long famine phase.

Microbial culture was able to fully consume both carbon sources supplied, glycerol and methanol. However, their consumption was not simultaneous. The glycerol fraction was preferably consumed with a high specific rate of 11.62 Cmmol Gly/gX.h until approximately 1 h during which DO concentration remained near zero. When glycerol was completely consumed, substantial increase of DO was observed, typical of the transition between feast and famine phases. At a significantly lower rate (0.49 Cmmol Meth/gX.h), methanol was consumed until exhaustion at 5.8 h, on the contrary to Moita *et al.* [66] work, in which methanol remained present along the entire cycle and accurate consumption rates were not possible to be determined. However, although PHB production was reported by Dobroth *et al.* [92] by a selected culture using exclusively the methanol fraction of CG, in this case, like in Moita *et al.* [66] work, methanol does not seem to contribute to polymer accumulation since after glycerol exhaustion, polymer production did not occur. Instead, his consumption may be associated with a separate microbial community, without the capacity to accumulate polymers that was able to coexist in the SBR.

A feast and famine regime was then established for glycerol consuming population where small feast phase extends for approximately 1 h before a long famine phase of 23 h (F/F ratio=0.04). During the feast phase, where polymer accumulation occurred, 80% of total carbon was consumed, while the remaining 20 % was consumed in the famine phase (as methanol).

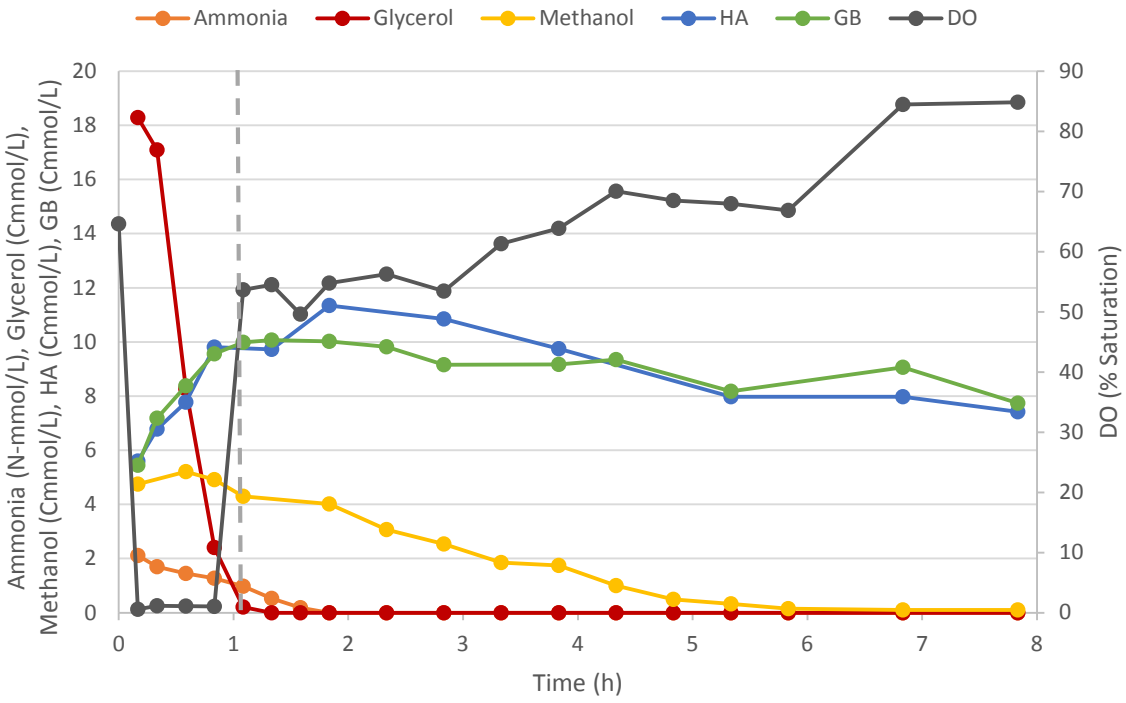


Figure 29 *- SBR2 cycle at 86th day of operation.*

Both PHA and GB accumulation occurred only in the first hour at the expense of glycerol consumption at similar specific accumulation rates, 0.05 Cmmol HA/Cmmol X.h and 0.06 Cmmol Gluc/Cmmol X.h respectively. Their yields were also very similar with values of 0.27 Cmmol HA/Cmmol S for PHA and 0.26 Cmmol Gluc/Cmmol S for GB. Although in this particular cycle PHA and GB accumulation parameter were almost identical, during the entire operation GB production yield and specific production rate was usually superior to PHA (**Figures 31-32**) with a less significant difference after day 86. Comparing to Moita *et al.* [66] work, although GB accumulation was also favored in relation to PHA, the difference between the two polymer was more significant, with a three times faster production rate and a twice as much yield.

It should be noted that monomer composition of PHA obtained at the end of feast phase was almost exclusively HB, with a very small fraction of HV (1.7%) which agrees with previous studies regarding the use of CG [66, 92].

Complete stoichiometric and kinetics parameters calculated for this particular cycle are presented in **Table 7**.

b) SBR performance

Since the beginning of operation, a clear feast and famine pattern was established in SBR2 with DO concentration decreasing right after feeding and suddenly increasing with carbon source depletion. Online data acquisition of DO concentration in the SBR allowed determining accurately F/F ratio of each cycle, with this evolution being used as indicator of inoculum adaptation. **Figure 30** shows the variation of the F/F ratio during the entire SBR2 operation.

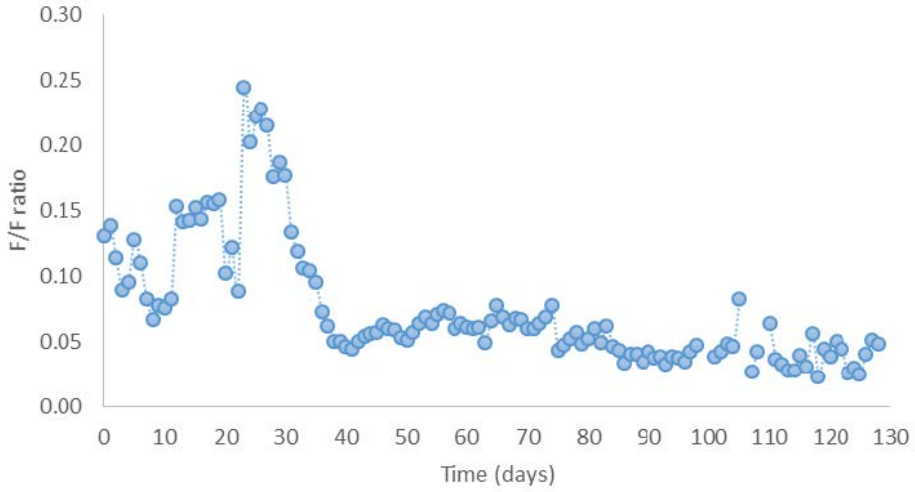


***Figure 30** - F/F ratio evolution during bacterial enrichment in SBR2.*

An initial period of some instability was observed until 38th day due to inoculum adaptation to the substrates and technical problems regarding the supply of correct feeding concentration. After that period, F/F ratio stabilized and was maintained between 0.02-0.08 for the remaining period of operation. F/F ratios reported in the literature that allow the selection of a culture with a predominantly storage response over growth are usually bellow 0.26 [61], which indicates that SBR2 was operated under appropriate condition to allow the PHA accumulating organisms to outcompete non-accumulating bacteria and thus being able to evidence a good storage response.

The performance evidenced by the microbial culture can be accessed in **Figure 31**, where it is shown both production (PHA and GB) and consumption (glycerol and methanol) specific rates during the operation time.

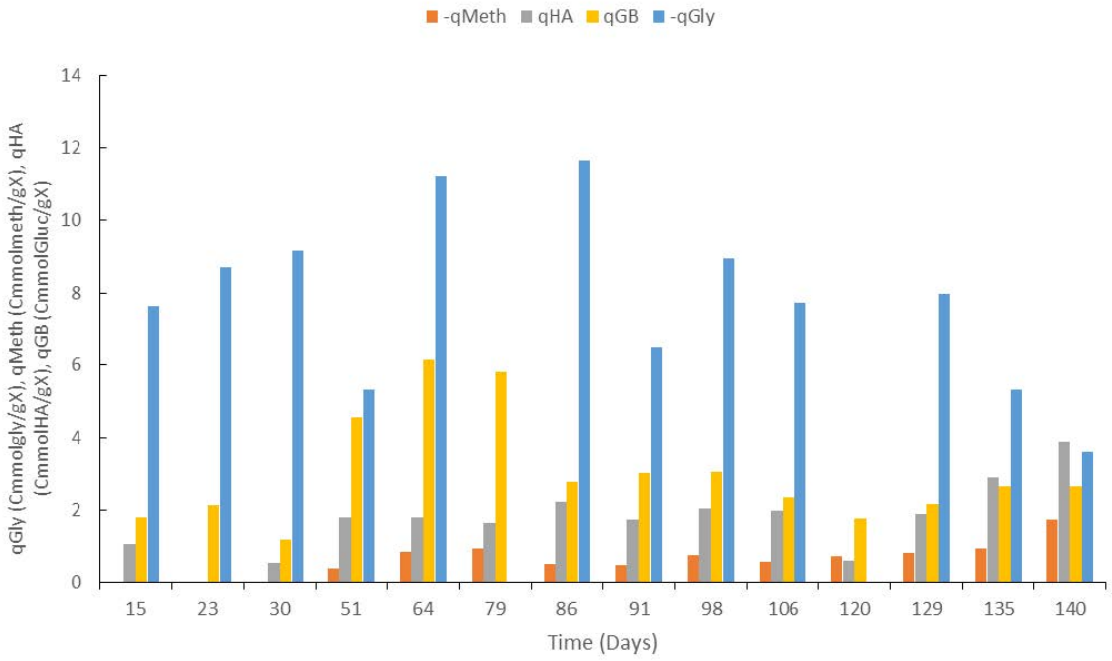


Figure 31 *- Evolution of specific production and consumption rates of the microbial culture in SBR2.*

In terms of carbon sources consumption, it is clear that glycerol was preferably consumed by the culture over methanol along the all operation. Moreover, methanol consumption was only possible to determine after 51st day. Before, methanol concentration remained unstable along the cycles without being possible to visualize a tendency of consumption. After that, methanol presented a relatively stable consumption rate reaching a maximum value of 0.95 Cmmol Meth/gX.h (day 79). Gycerol consumption however, had an initial increase until day 64 where it reached a maximum value (11.19 Cmmol Gly/gX.h) and then progressively decreased to similar rates registered at the beginning of operation. Glycerol consumption rate behavior was followed by GB accumulation rate, which also reached his maximum value at day 64 (6.13 Cmmol Gluc/gX.h) and then decreased continuously. This behavior similarity can be explained by the fact that GB accumulation is more efficient than PHA, as demonstrated by Dirks *et al.* [117]. So a higher substrate consumption rate would allow a higher production rate of the more easily accumulated polymer.

PHA accumulation on the other hand, revealed an initial increase until day 51, and then remained fairly stable with a maximum of 0.051 Cmmol PHA/CmmolX.h being reached

at day 86. This value is slightly higher to the one obtained by Moita *et al.*[66] (0.04 Cmmol HB/Cmmol X.h), using the same feedstock.

Considering **Figures 30-31**, there were not significant changes in the analyzed parameters occurred since day 91, and from this moment, the system seem to have reached a steady-state. This corresponds to a stabilization period of 10.2 SRT which is similar to the 8.5 SRT obtained by Moita *et al.* [66]. However a shorter stabilization period was expected since the inoculum used was originated from a previously PHA-accumulating culture selected with CG. In spite of that, the culture selected by the imposed operational conditions, was able to evidence, after day 91, preferred polymer storage response over growth (**Figure 32**).

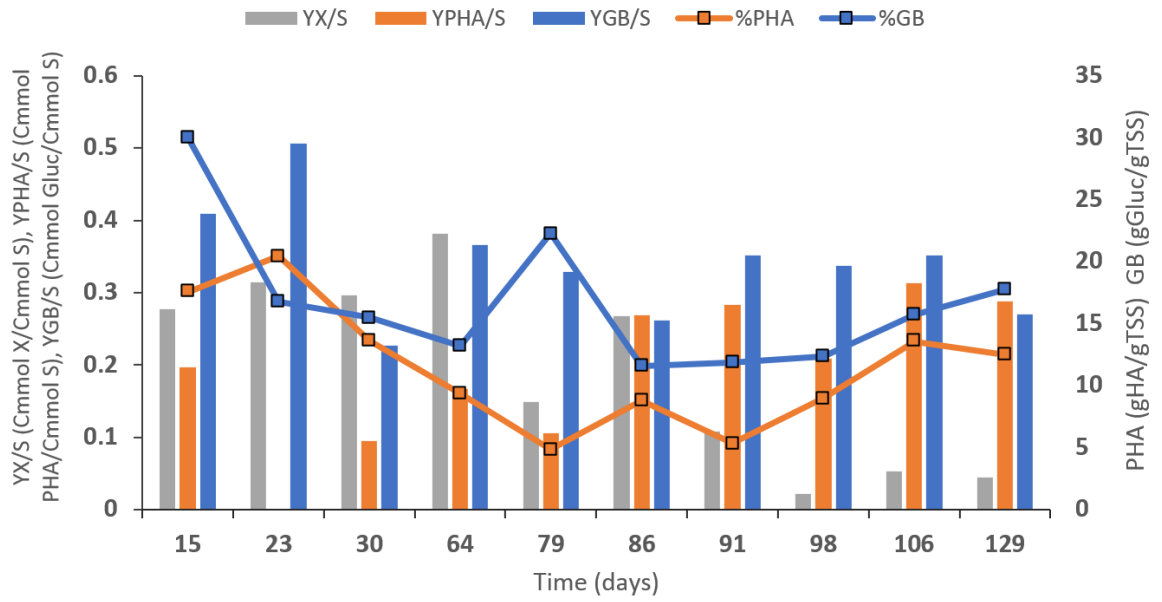


Figure 32 *- Evolution of biomass and biopolymers production yields and biopolymers maximum content in SBR2.*

Moreover, during culture enrichment biomass production yield decreased substantially, while PHA production yield increased, which demonstrates the enrichment of the microbial culture in PHA accumulating organisms obtained.

An average PHA production yield of 0.21±0.08 Cmmol PHA/Cmmol S and PHA content of 11.5±5.01% were obtained during the culture acclimatization with respective maximum values of 0.31 Cmmol PHA/Cmmol S (day 106) and 20.43% (day 23). Similar average PHA production yield was obtained by Moita *et al.* [66] although lower PHA content percentages

were registered. Average kinetic and stoichiometric parameters calculated from analyzed cycles are summarized in **Table 7**.

Table 7 *- Kinetic and stoichiometric parameters obtained for the SBR2.*

Assay	q_{Gly}	q_{Meth}	q_{HA}	q_{GB}	$\%HA_{max}$	$\%GB_{max}$	$Y_{HA/S}$	$Y_{GB/S}$	$Y_{X/S}$
Average daily cycles	8.47 (1.92)	0.66 (0.22)	1.58 (0.49)	3.20 (1.62)	11.49 (5.01)	16.70 (5.71)	0.21 (0.08)	0.34 (0.08)	0.19 (0.13)
Cycle day 86	11.62	0.49	2.24	2.80	8.75	11.59	0.27	0.26	0.27
Accumulation					**day 42**				
1º pulse	10.15	nd	1.85	6.05	10.21	32.00	0.47	0.74	-
2º pulse	6.97	nd		-	15.83	37.99	0.24	-	-
3º pulse	7.08	nd		-	21.39	37.71	0.24	-	-
4º pulse	3.77	nd		-	25.70	38.08	0.43	-	-
5º pulse	4.79	nd	-	-	24.94	37.55	-	-	-
Accumulation					**day 58**				
1º pulse	6.93	nd	4.00	5.82	15.20	31.21	0.40	0.73	-
2º pulse	5.80	nd		-	25.79	36.77	0.54	-	-
3º pulse	5.77	nd		-	37.29	35.21	0.62	-	-
4º pulse	4.00	nd	-	-	37.37	33.93	-	-	-
5º pulse	3.99	nd	-	-	36.52	33.97	-	-	-

(standard deviation); (nd) - not determined.
q_{Gly} (Cmmol Gly/gX.h); q_{Meth} (Cmmol Meth/gX.h); q_{HA} (Cmmol HA/g X.h); q_{GB} (Cmmol Gluc/gX.h).
$\%HA_{max}$ (% gHA/g TSS); $\%GB_{max}$ (% g Gluc/g TSS).
$Y_{X/S}$ (Cmmol X/Cmmol S); $Y_{HA/S}$ (Cmmol HA/Cmmol S); $Y_{GB/S}$ (Cmmol Gluc/Cmmol S).

4.2.2 Accumulation Assays

The SBR2 microbial population was tested for its accumulation capacity by performing two accumulation assays with a multiple pulse addition feeding strategy in order to avoid a potential substrate inhibition. **Figures 33-34** present the results obtained

in such accumulation assays performed at 42nd and 58th day of operation respectively. Kinetic and stoichiometric parameters of both assays are listed in **Table 7.**

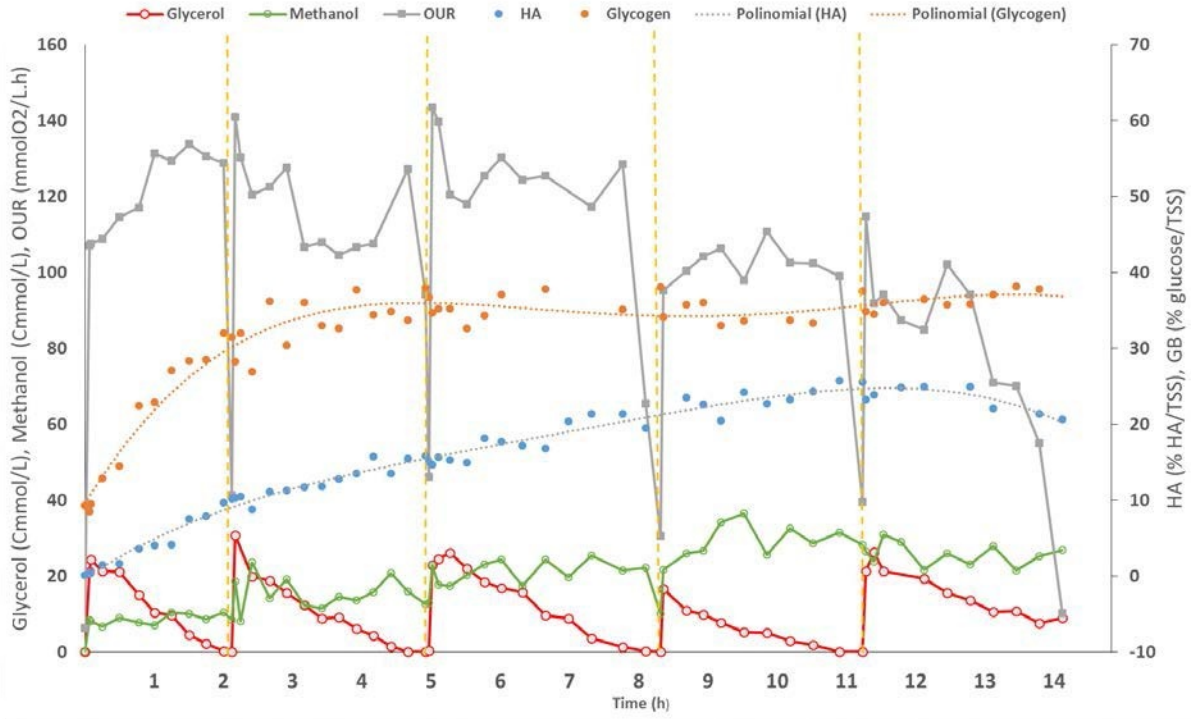


Figure 33 *- Accumulation assay of SBR2 performed at 42nd day of operation with yellow dashed lines indicating the moments of pulses addition.*

Regarding the assay performed at day 42 (**Figure 33**), it can be seen that it starts with the highest glycerol consumption rate (10.15 Cmmol Gly/gX.h) in the first pulse, in which the most part of GB is produced (6.05 Cmmol Gluc/gX.h). At the same time, PHA accumulation also occurred, and it was extended until the end of the 4th pulse in a much lower rate than GB (1.85 Cmmol HA/g X.h), reaching a maximum content of 25.7%. As seen before, glycerol was clearly preferably consumed by the culture while methanol seemed to accumulate along the assay. As seen in previously in SBR performance methanol was consumed by the culture at a much lower rate than glycerol, which means that the time between cycles was not enough to allow the culture to consume it completely. This led to its accumulation along the pulses. As hypothesized by Moita *et al.* [66], the high concentration of methanol in the reactor medium may have a toxic effect in MMC, which can be responsible for accumulation stoppage at the end of the fourth pulse and thus limiting the accumulation capacity of the culture.

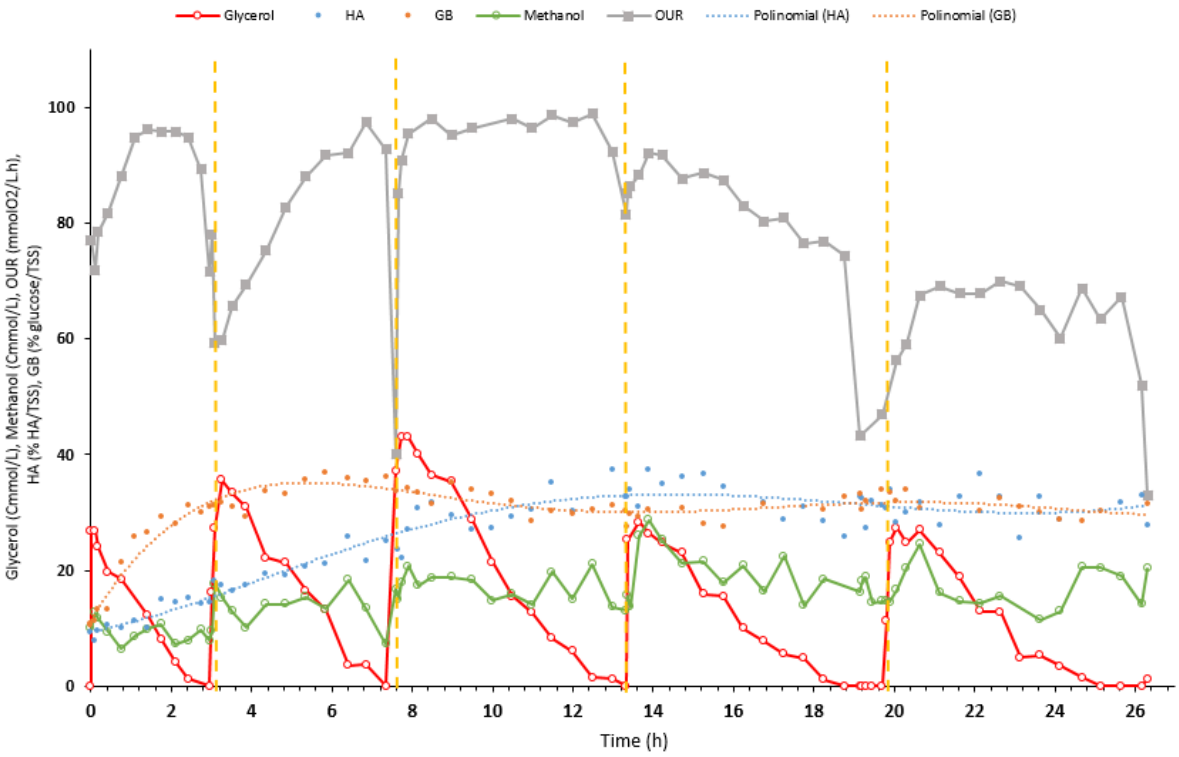


Figure 34 *- Accumulation assay of SBR2 performed at 58th day of operation with yellow dashed lines indicating the moments of pulses addition.*

In the second assay performed (**Figure 34**), some changes were verified. Specific glycerol consumption rate started with a lower maximal consumption of 6.93 Cmmol Gly/gX and showed a decreasing tendency along the assay reaching the lowest value (3.99 Cmmol Gly/gX.h) in the last pulse. Similar methanol concentration profile was obtained as in the previous test. GB and PHAs were produced at different velocities and to different extents. While GB was essentially produced in the first pulse like in the first assay, with a specific rate of 0.132 Cmmol/CmmolX.h, PHA was produced at a slower velocity (0.090 Cmmol/CmmolX.h) but only until the end of the third pulse. In terms of maximum content, PHA and GB presented a similar percentage of 37.4% (Δ 28.1%) and 36.8% (Δ 26.1%) respectively. Comparing to the first assay, while GB reached similar contents, PHA maximum content of the culture improved. Using CG and performing an assay with the same feeding strategy, Moita *et al.* achieved a slightly higher PHA content of 46.91% but with a lower specific rate (0.03 Cmmol/CmmolX.h) and GB maximum content of 16.69% with an identical specific rate of 0.131 Cmmol Gluc/Cmmol X.h [66]. Comparing the two works: GB accumulation behavior was similar with a fast accumulation at the beginning of

the assays followed by a slow consumption. In the case of PHA, in this study, its accumulation was faster but stopped at the end of third pulse while in Moita *et al.,* PHA was accumulated continuously until the end of the assay at a lower rate.

Other accumulation assays should have been performed especially in a later period of SBR operation where the MMC presented a more stable performance and where PHA production yield was higher which could have led to higher PHA contents.

Few other works reported the production of PHA using MMC and real waste substrate with non-VFA organic matter. Some known examples are Gurieff [124], using primary sludge as the feedstock obtained a PHA content of 20% (cdw) and 39% with fruit cannery wastewater; Liu *et al.* [54] reported a PHA content of 20% (cdw) using tomato cannery wastewater; Moralejo-Gárate *et al.* [91]obtained up to 80% (cdw) from pure glycerol and Dobroth *et al.* [92] also using CG obtained a PHB content (62% cdw).

4.2.3 Microbial Community Analysis

With the purpose of optimizing the reactor operation and the efficiency of the process, the study of the microbial community is required. In this investigation, the bacterial community selected in the SBR2 was morphologically characterized along the operational time. The predominant morphotypes were preliminary described by phase contrast microscopic analysis while the role of each morphotype in PHA storage was shown by Nile Blue A staining, a specific staining for lipidic inclusions.

Since the beginning of operation under ADF condition, morphology of the culture showed a perceivable predominance of bacteria arranged in tetrads. In this aggregates, it was easily perceptible the presence of inclusions bodies of PHA by the presence of brilliants dots in the cells after Nile Blue A staining procedure under epiflurescence. **Figure 35** refers to a sample of day 51 that clearly shows the presence and dominance of such morphology.

Tetrad-forming-organisms morphology is commonly described in other PHA accumulating enrichment works such as Pisco *et al.* [125], which in that case FISH analysis showed these were TFOmix and DFmix binding cells and thus belonging to *Defluvicoccus* genus.

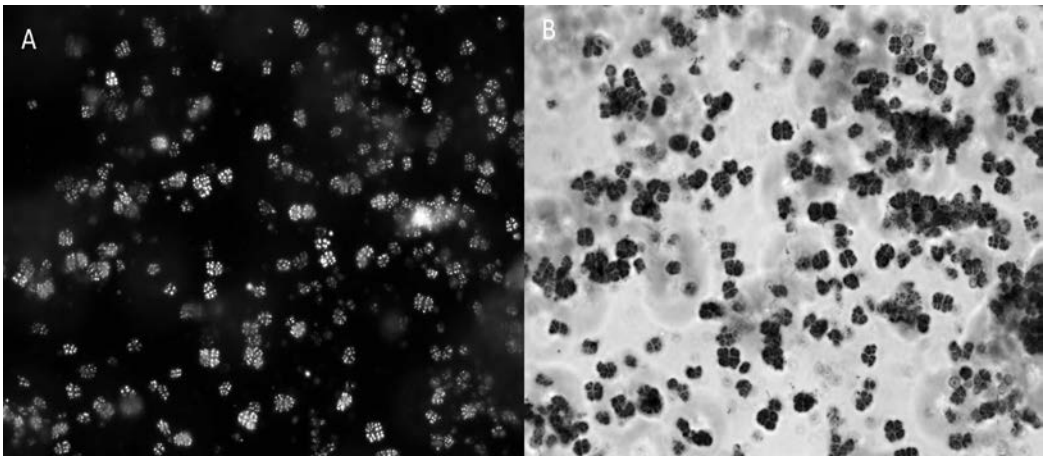


Figure 35 *- Image of sample collected at the end of feast phase of cycle of day 51 subjected to Nile Blue A staining (A) and under phase contrast (B). Ampliation 400x.*

Along culture enrichment process, culture morphology composition suffered some change. In **Figure 36**, a sample of the end of SBR2 operation (day 120), a more diverse morphology composition was present.

Besides tetrad-forming-organisms that remain in majority, coccobacilli shaped bacteria with PHA accumulation capacity were also present. In fact, in this sample, the fluorescence observed in the cells with this morphology appears to be more intense than the tetrads, which suggests that they have a higher accumulation capacity. Therefore, comparing to the initial morphology of the culture, the imposed operational conditions on the SBR have led to an enrichment of coccobacilli shaped bacteria with a high PHA accumulation capacity which is in accordance with SBR performance improvement verified.

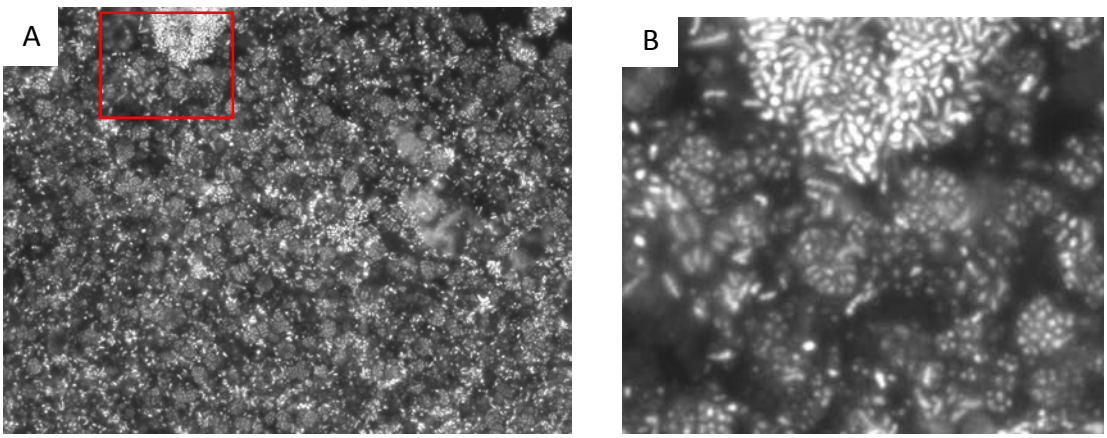


Figure 36 *- Image of sample collected at the end of feast phase of cycle of day 120 subjected to Nile Blue A staining (400x) (A) and amplification showing detail (B).*

5

CONCLUSIONS

The operation of SBR1 for 97 days under aerobic dynamic feeding with HSSL as a substrate was not able to select a MMC with a stable performance. The inoculum was able to adapt to a feedstock quite diverse in its composition, by consuming acetate and xylose but not the main carbon source, lignosulphonates. However the accumulation of both PHA and GB was observed. The imposed operational conditions resulted in a partial selection of the culture, since only the acetate consuming organisms experienced a real feast/famine regime, while xylose remained present almost through the entire cycle. Consequently, the resultant culture turned out to be more specialized in the production of GB instead of PHA with an average content of 18.32 ± 4.14% and 4.31 ± 1.18% and an average production yield of 0.34 ± 0.12 Cmmol Gluc/Cmmol S and 0.20 ± 0.13 Cmmol HA/Cmmol S, respectively, during the selection stage. The results of the accumulation assay performed were below expectations with a maximum PHA content of 4.6%, which was most likely due to the assay conditions that were not the most appropriate.

The FISH analysis of selected microbial community revealed the dominance of *Alphaproteobacteria*, in which *Paracoccus* and *Rhodobacter* genus were identified, but also the existence of *Betaproteobacteria, Gammaproteobacteria* and bacteria belonging to the classes *Flavobacteria, Bacteroides Sphingobacteria*. DGGE analysis confirmed the inefficiency of the system in selecting a homogenous culture.

Although experimental results allowed for a proof of concept of PHA production by mixed cultures using HSSL as substrate, significant optimization is needed in order to stabilize the population and obtain a sufficiently productive process to be implemented.

The culture enrichment process of SBR2 for 129 days using CG as substrate turned out to be more efficient with a relatively stable MMC being obtained after day 51. Culture consumed both glycerol and methanol fractions of CG (although it seemed that only

glycerol accounted for PHA accumulation) and was able to accumulate both PHB and GB in similar amounts. Accumulation tests achieved a considerable PHB content (37.4%), which was in the middle range of similar works reported in the literature. The fact that crude glycerol does not need a pre-fermentation step to be converted into PHB makes the overall production process economically more sustainable when compared other processes that require a pre-fermentation step.

6

FUTURE WORK

Regarding SBR1, this system is still far from being efficient, which means that several operational conditions should be further studied and optimized: substrate concentration and composition, organic loading rate (OLR), solids retention time, carbon to nitrogen ratio, pH and cycle length. Since HSSL is a complex mixture, a detailed profile of the different compounds and secondary metabolites formed along the SBR cycle should be determined by GC-MS in order to evaluate their potential toxic effect to MMC. Also the role of the different compounds present in the HSSL (xylose, glucose and lignosulphonates) to the PHA and GB production should be determined such as the physical characterization of the polymer obtained by the MMC.

In order improve the utilization of the high carbon content of HSSL and at the same time provide a more suitable substrates for PHA accumulation, a pre-treatment step consisting in the acidogenic fermentation of HSSL could be applied in order to improve the PHA content and lower GB.

In terms of microbial community characterization, it should be further investigated regarding the taxonomic composition, since only few genera were identified. Sequencing of DGGE bands would allow identifying the dominant species present in the culture.

As for SBR2 system, although it showed already to be capable of achieving reasonable PHA contents, further efforts to optimize the system should be made in order to improve its capacity before scaling-up. First of all, the potential toxic effect to the MMC of methanol in higher concentrations should be investigated and then proceed to increase substrate concentration or decrease cycle length in order to achieve an improved culture selection. Besides that, the microbial community composition should be further investigated regarding molecular techniques such as FISH and PCR-DGGE.

7

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